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Award Number: DAMD17-02-2-0012

TITLE: Low Level Exposure to Sulfur Mustard: Development of a
SOP for Analysis of Albumin Adducts and of a System for
Non-Invasive Diagnosis on Skin

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REPORT DATE: December 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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DOCUMENTATION PAGE

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|---|---|--|---|
| 1. AGENCY USE ONLY (Leave blank) | | 2. REPORT DATE December 2003 | 3. REPORT TYPE AND DATES COVERED Annual (1 Dec 2002 - 30 Nov 2003) |
| 4. TITLE AND SUBTITLE Low Level Exposure to Sulfur Mustard: Development of a SOP for Analysis of Albumin Adducts and of a System for Non-Invasive Diagnosis on Skin | | | 5. FUNDING NUMBERS DAMD17-02-2-0012 |
| 6. AUTHOR(S) Daan Noort, Ph.D Dr. Govert P. van der Schans Dr. Hendrik P. Benschop | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) TNO Prins Maurits Laboratory 2280 AA Rijswijk, The Netherlands E-Mail: noort@pml.tno.nl | | | 8. PERFORMING ORGANIZATION REPORT NUMBER |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | 10. SPONSORING / MONITORING AGENCY REPORT NUMBER |
| 11. SUPPLEMENTARY NOTES | | | |
| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | 12b. DISTRIBUTION CODE |
| 13. ABSTRACT (Maximum 200 Words) In the second year of the grant period, the persistence of the various sulfur mustard adducts has been studied, in particular the adduct to the Cys-34 residue in albumin. After exposure of rats (0.3 mg/kg, i.v.), the tripeptide adduct (S-HETE)Cys-Pro-Tyr could be determined until 7 days after the exposure; the observed half-life time of sulfur mustard - alkylated rat albumin was 2 days, which is in accordance with literature values. In the corresponding globin samples of these animals, the N-terminal valine adduct could still be determined after 28 days after the exposure. Remarkably, the maximum adduct level was reached after 2-3 days, implicating the presence of intact sulfur mustard in the animal during the first 2-3 days. With regard to the most abundant adduct, i.e., the histidine adduct, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared in the first year of the grant period. In the second year of the grant period it turned out that the 5/6-carboxyfluoresceine (FAM) group gives the best results with regard to sensitivity. The FAM derivatives could be determined at a level of approximately 25 pg/ml. Results obtained thus far indicate that the albumin - tripeptide assay will become the method of choice for development into an SOP. A tentative Standard Operating Procedure has been drafted for this assay, that incorporates the earlier developed method for rapid isolation of albumin by affinity chromatography and the use of a deuterated internal standard. An immunoslotblot assay was developed for detection of sulfur mustard adducts to keratin in the first year of the agreement, enabling the detection of <i>in vitro</i> exposure of human callus to 0.2 μ M sulfur mustard. Problems were encountered regarding the reproducibility of the assay when isolated keratin was used; this is currently under investigation. | | | |
| 14. SUBJECT TERMS Adducts, albumin, biomonitoring, GC-MS, hemoglobin, human blood, immunochemical detection, immunoslotblot, keratin, LC-tandem MS, retrospective detection, skin, sulfur mustard | | | 15. NUMBER OF PAGES 51 |
| | | | 16. PRICE CODE |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT Unlimited |

FOREWORD

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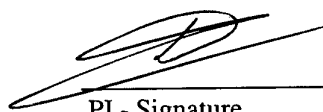
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ACKNOWLEDGMENTS

Many thanks are due for the crucial contributions of Alex Fidder and Mrs. Saskia de Kant who were primarily responsible for the organic chemistry and the analytical chemistry, and to Mrs. Roos Mars and Mrs. Helma van Dijk for the biochemistry and immunochemistry, as described in this report. The authors are grateful to Albert Hulst, Mrs. Carla Degenhardt, Ad de Jong, Ben van Baar and Eric Wils who carried out the mass spectrometric analyses, to Simon van Krimpen who did the NMR analyses, and to Cees van der Wulp for his assistance to quantitative immunofluorescence microscopy experiments.

SUMMARY

The need for retrospective detection procedures for exposure to low levels of chemical warfare agents has been urgently illustrated by the conflicts in the Gulf Area and, especially, in the attempts to clarify the Gulf War Syndrome. The research within the current cooperative agreement (DAMD17-02-2-0012) aims at:

1. development of a mass spectrometric or fluorescence-based method for retrospective detection of exposure to low doses of sulfur mustard, based on improvement of analysis of an adducted tripeptide in albumin and of adducts to histidine in hemoglobin and albumin (**part A, B and C** of revised statement of work).
2. development of immunoslotblot assays for quantitation of protein – sulfur mustard adduct levels, using monoclonal antibodies already available from previous research in this field (**part D** of revised statement of work).

1. The albumin assay (**part A**) is based on our previous finding that upon pronase treatment of sulfur mustard alkylated albumin, a tripeptide cysteine(S-2-hydroxyethylthioethyl)-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) results which has favorable mass spectrometric properties.

In the second year of the agreement, animal experiments have been conducted in order to study the persistence of the albumin – sulfur mustard adduct *in vivo*. From the research conducted in the first year of the agreement, it was found that after pronase treatment of rat albumin, isolated from rat blood exposed to [¹⁴C]-labelled sulfur mustard *in vitro*, the tripeptide (S-HETE)Cys-Pro-Tyr could be analyzed in an analogous way as performed for (S-HETE)Cys-Pro-Phe formed after pronase treatment of human serum albumin. The relative level of formed (S-HETE)Cys-Pro-Tyr in rat albumin was determined to be 5% of the total amount of alkylation, which is more or less similar to alkylation of human serum albumin. In the second year of the grant period, rats were challenged (i.v.) with 0.3 mg/kg sulfur mustard which corresponds to approximately 0.1 LD₅₀. The animal experiments show that the tripeptide (S-HETE)Cys-Pro-Tyr is formed *in vivo*, and that it can be determined at least 7 days after exposure. On the other hand, the adduct to the N-terminal valine residue in rat hemoglobin could be determined even after 28 days after the exposure. This large difference can be explained by the very high turn-over rate of albumin in rats, which is much higher than in humans (half – life times 2-3 and 16 days, respectively). Remarkably, in case of hemoglobin adducts it was observed that the maximum adduct level was reached only after 2-3 days after the exposure. This implicates that intact sulfur mustard is still present in the animal at least 2-3 days after the exposure.

With regard to analysis of the histidine-sulfur mustard adducts (i.e., the most abundant amino acid adducts formed after exposure to sulfur mustard), a work-up procedure was developed in the first year of the cooperative agreement for isolation of these adducts from amino acid mixtures resulting from acidic hydrolysis of globin or albumin (**part B**). This procedure afforded purified histidine adducts, which could be further derivatized, inter alia with fluorescent labels for detection with laser induced fluorescence. Thus, for detection with laser-induced fluorescence (LIF), various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared in the first and second year of the grant period. Although the sensitivity for capillary electrophoresis – LIF analysis of the 5/6-carboxyfluorescein (FAM) derivative of N1/N3-HETE-histidine is satisfactory (20-25 pg/ml), it remains to be seen that the method is suitable for analysis of complex mixtures, i.e., amino acid mixtures resulting from acidic or enzymatic hydrolysis of albumin and/or hemoglobin. This will be further investigated in the third year of the grant period.

Based on the results obtained thus far the tripeptide assay looks much more promising than the histidine adduct assay. A tentative Standard Operating Procedure (**part C**) has been drafted for the albumin tripeptide assay, on the basis of the experiments performed in the first and second year of the grant agreement.

2. With regard to the development of immunoslotblot assays for detection of sulfur mustard adducts to proteins (**part D**), several clones are available producing antibodies which show specificity not only for hemoglobin alkylated with sulfur mustard (50 μ M) but also for alkylated keratin. In the first year of the cooperative agreement, an improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin. Also, an immunoslotblot assay was set up in the first year for detection of sulfur mustard adducts to keratin. Up to now, the lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was at 25 fmol adducted sulfur mustard using 0.5 μ g keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/ 5×10^7 unadducted amino acids. The minimum detectable concentration for *in vitro* treatment of human callus with sulfur mustard was 0.2 μ M. Problems were encountered regarding the reproducibility of the assay when isolated keratin was used; this is currently under investigation. A complicating factor appeared to be the fact that contamination of the isolated keratins with small amounts of SDS (essential for extraction of keratin) interfered with the binding of these keratins in the immunoassays which might result in erroneous outcomes.

In summary, the following technical objectives, as described in the revised Statement of Work have now been completed after the second year of the grant period:

Part A: Mass spectrometric or fluorescence-based analysis of an adducted peptide from albumin
t.o. 1, 2, 3, 4, 5, 8 and 9 (partly)

Part B: Analysis of N1/N3-histidine
t.o. 10, 11, 12 (partly)

Part C: Development of a standard operating procedure
t.o. 17 (tentative)

Part D: Development of immunochemical assays to quantify the adduct levels of sulfur mustard to proteins
t.o. 19, 20 (partly), 21

N.B.

Originally proposed time schedule for revised statement of work

| | | |
|-----------------------|----------------------|---|
| 1 st year: | technical objectives | 1, 2, 3, 4, 6, 10, 19, 20 |
| 2 nd year: | technical objectives | 5, 7, 8, 11, 12, 20, 21 |
| 3 rd year: | technical objectives | 9, 13, 14, 15, 16, 17, 18, 20, 22, 23, 24 |

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I INTRODUCTION

The use of chemical warfare agents in the Iran-Iraq war has learned that reliable methods for identification and verification of exposure to chemical warfare agents in alleged casualties were urgently needed, and not available at that time. Furthermore, experience with the casualties in the Iran-Iraq war and with other incidents learned that biopsies or autopsies of alleged victims often become available several days or even weeks after alleged exposure. Recently, the need for retrospective detection of exposure and, even more demanding, of low level exposure has been vividly illustrated in the attempts to clarify the causes of the so-called "Persian Gulf War Syndrome". Quantitation of low level exposure provides an indispensable basis to study the subtle toxic effects of such exposures. Moreover, the application of reliable procedures to exclude that even trace exposure to chemical agents has occurred will contribute to combat readiness of the soldier.

Within the framework of previous grants (DAMD17-88-Z-8022, DAMD17-92-V-2005 and DAMD17-97-2-7002) we have worked on the development of methods for diagnosis and dosimetry of exposure to sulfur mustard (1-16). Our approach is based upon the development of monoclonal antibodies against adducts of sulfur mustard with DNA and proteins for use in a variety of immunochemical assays and upon the development of procedures for mass spectrometric analysis of the adducts. The immunochemical assays can be performed on small samples, are highly sensitive, and can be applied "on site" when properly developed. Results obtained from the GC-MS or LC-MS-MS analyses will confirm the immunochemical results and will provide information on the structure of the adducts. In this way, it can be established whether casualties have indeed been exposed to sulfur mustard, whereas dosimetry of the exposure will be a starting point for proper treatment of the intoxication.

The main advantage of detection of adducts of sulfur mustard in proteins over those to DNA is the expected much longer half-life of protein adducts (17). Whereas in DNA of human skin most of N7-(2-hydroxyethylthioethyl)-guanine (N7-HETE-Gua)¹ has been removed two days after *in vivo* exposure (18), it is expected that adducts to proteins have life-spans varying from several weeks to months. Consequently, the retrospectivity of the diagnosis in protein adducts is superior to that in DNA. Moreover, detection is supposedly also more sensitive in case of single, protracted, and intermittent exposure to sulfur mustard at low concentrations, since the protein adducts will accumulate.

Explorative research within the context of our previous grant DAMD17-97-2-7002 has yielded important clues for development of new SOPs that satisfy the abovementioned needs. Firstly, we have lowered the minimum detectable concentration of sulfur mustard in human blood by at least one order of magnitude by mass spectrometric analysis of adducted peptides from enzymatically digested albumin (11). Secondly, we have found that, as in the case of hemoglobin, adducts to histidine are by far the most abundant adducts in albumin. Thirdly, we have recently succeeded to raise monoclonal antibodies against sulfur mustard adducts to hemoglobin, albumin and keratins. The latter opens the possibility to detect *in vivo* skin exposure to sulfur mustard in a non-invasive way under operational conditions, *e.g.*, by means of immunofluorescence techniques. If these qualitative immunoassays indicate that exposure to sulfur mustard has occurred, adduct levels have to be quantified in order to estimate the extent of sulfur mustard injury.

Consequently, we decided to continue our investigations on four topics, within the framework of the current Cooperative Agreement DAMD17-02-2-0012:

- A. further development of the mass spectrometric analysis of the tripeptide (S-HETE)Cys-Pro-Phe, resulting from pronase digestion of albumin alkylated by sulfur mustard (t.o. 1-9; year 1-2).
- B. perform further research on analysis of the most abundant adduct formed after exposure of proteins to sulfur mustard, *i.e.*, N1/N3-HETE-histidine (t.o. 10-13; year 1-2).

¹ HETE: 2-(hydroxyethyl)thioethyl

- C. develop the most suitable procedure into a SOP (t.o. 14-18; year 2-3)
- D. develop immunochemical assays in order to quantify levels of sulfur mustard adducts to proteins (t.o. 19-24; year 1-3).

In the first year of the current Cooperative Agreement (DAMD17-02-2-0012) the procedure for isolation of albumin from human blood could be substantially shortened by using affinity chromatography. The lowest detectable exposure level was improved by one order of magnitude (1 nM) by work-up of 20 mg amounts of albumin. Furthermore, the use of an internal standard has been worked out. After i.v. administration of sulfur mustard to the marmoset, the specific (S-HETE)Cys-Pro-Phe adduct could be analyzed after pronase digestion of albumin, for at least 28 days after the challenge. Furthermore, a work-up procedure was developed for rapid isolation of the histidine adducts from amino acid mixtures resulting from acidic hydrolysis of globin or albumin. Derivatization of the histidine adducts with trifluoroacetic acid anhydride for GC-MS analysis resulted in the formation of a tris(trifluoroacetyl) derivative with favorable mass spectrometric properties. For detection with laser-induced fluorescence, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared. An immunoslotblot assay was developed for detection of sulfur mustard adducts to keratin. The lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was at 25 fmol adducted sulfur mustard using 0.5 µg keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/ 5×10^7 unadducted amino acids. The minimum detectable concentration for in vitro treatment of human callus with sulfur mustard was 0.2 µM.

In the second year of the cooperative agreement work on all 4 subjects was continued.

II MATERIALS AND INSTRUMENTATION

II.1 Materials

WARNING: Sulfur mustard is a primary carcinogenic, vesicant, and cytotoxic agent. This compound should be handled only in fume cupboards by experienced personnel.

Technical grade sulfur mustard was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5%. The following compounds were synthesized as described previously: N α -Fmoc-(N1/N3-HETE)histidine (9) and sulfur mustard-*d*₈ (4)

The following commercially available products were used:

Fluorescein-5-isothiocyanate (FITC 'isomer I'), 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA), 5/6-carboxyfluorescein succinimidyl ester (FAM, SE), and Oregon Green 488-X succinimidyl ester 6-isomer (all Molecular Probes Europe BV, Leiden, The Netherlands). Diethanolamine, acetonitrile (Baker Chemicals, Deventer, The Netherlands); human serum albumin, pentafluorobenzyl bromide (HSA), (Fluka, Buchs, Switzerland); d,l-dithiothreitol (DTT), iodoacetic acid sodium salt, TPCK trypsin, bovine serum albumin, human hemoglobin, pronase Type XIV from *Streptomyces Griseus* (E.C. 3.4.24.31), tween 20 (polyoxyethylenesorbitan monolaurate) (Sigma Chemical Co., St. Louis, MO, U.S.A.); immobilized TPCK-trypsin (14 units/ml gel) heptafluorobutyrylimidazole (Pierce, Rockford, IL, U.S.A.); 9-fluorenylmethylchloroformate (Fmoc-Cl), β -mercaptoethanol, Tris.HCl, EDTA (Janssen, Beerse, Belgium); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PyBOP (Novabiochem); trifluoroacetic acid, thiodiglycol (Aldrich, Brussels, Belgium); fetal calf serum (FCS; LCT Diagnostics BV, Alkmaar, NL); goat-anti-mouse-Ig-alkaline phosphatase, goat anti-mouse-IgG-alkaline phosphatase (KPL, Gaithersburg, USA); microtiter plates (96 wells; polystyrene 'high binding'), microtiter plates (96-wells culture plates) (Costar, Badhoevedorp, The Netherlands); 4-methylumbelliferyl phosphate (MUP), dispase, RNase T1, protease inhibitor cocktail mini tablets, (Boehringer, Mannheim, Germany); rabbit-anti-mouse-Ig-horse radish peroxidase (Dakopatts, Glostrup, Denmark); FITC-labeled 'goat-anti-mouse' (Southern Biotechnology Associates, Birmingham, AL), RPMI-1640 medium (Gibco BRL, Breda, The Netherlands); skimmed milk powder, less than 1% fat, (Campina, Eindhoven, The Netherlands). Carbosorb and Permablend scintillation cocktail were obtained from Canberra Packard (Tilburg, The Netherlands).

Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10 kDa filters were obtained from Schleicher & Schuell (Dassel, Germany). SepPak Florisil and SepPak C-18 cartridges were obtained from Waters (Bedford, MA). Centrex UF-2 (3 or 10 kDa molecular weight cut-off) centrifugal ultrafilters were procured from Schleicher & Schuell (Keene, NH). Ultrafree (100 kD molecular weight cut-off; 15 ml) centrifugal ultrafilters were obtained from Millipore (Bedford, MA). Albumin affinity chromatography was carried out on HiTrap Blue HP columns (1 ml; Amersham Biosciences, Uppsala, Sweden). Desalting of albumin fractions was carried out on PD-10 columns containing Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden). Dowex 50WX8 was obtained from Fluka.

Venous blood of human volunteers (10 ml, with consent of the donor and approval of the TNO Medical Ethical Committee) was collected in evacuated glass tubes, containing Na₂EDTA (15 mg).

Human callus was obtained from chiropodists. Human skin resulting from cosmetic surgery was obtained from a local hospital with consent of the patient and approval of the TNO Medical Ethical Committee.

II.2 Instrumentation

UV absorbance and UV spectra were recorded on a UV/VIS Spectrometer, Lambda 40 (Perkin Elmer, Breda, The Netherlands).

Microtiter plates were washed using the Skanwasher 300 (Skatron Instruments, Norway; Costar). The fluorescence on microtiter plates (excitation at 355 nm; emission at 480 nm) was recorded with a Cytofluor II (PerSeptive Biosystems, Framingham, MA).

Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 mm² slots) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). Protein was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, The Netherlands). An Enhanced Chemiluminescence Blotting Detection System (Boehringer) was used for the detection of peroxidase activity. The chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

Liquid chromatography experiments were run on an ÅKTA explorer chromatography system (Amersham Pharmacia, Uppsala, Sweden). Columns used were a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden), a Zorbax SB C-18 column (4.6 mm x 150mm; 5 µm, Zorbax, Mac-Mod Analytical, Chadds Ford, PA, USA) and a Source 15 RPC column (Amersham Pharmacia, Uppsala, Sweden).

LC/electrospray tandem mass spectrometric experiments were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C18 (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H₂O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10⁻⁴ mBar).

Other LC/electrospray tandem MS spectra were recorded on a VG Quattro II triple quadrupole mass spectrometer (Micromass, Altrincham, U.K.). The analyses were carried out with multiple reaction monitoring at a dwell time of 2 s, unless stated otherwise. Operating conditions were: capillary voltage 3.6 kV, cone voltage 25 V, collision energy 15 eV, gas (argon) cell pressure 0.3 Pa, and source temperature 120 °C.

Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was performed on a Beckman P/ACE 5000 CE instrument equipped with a Laser Induced Fluorescence detector (Fullerton, CA, USA). Excitation wavelength was 488 nm, emission wavelength 520 nm. The fused silica capillary (i.d. 75 µm) was purchased from Composite Metal Services (The Chase, Hallow, Worces., UK). The length of the capillary was 47 cm (40 cm effective length to the detector). Sample introduction was performed by pressure injection for 20 s. Separation voltage was 20 kV. The buffer used consisted of 20 mM Borax, pH 9. Radioactivity countings were performed on a Packard Tri-Carb series Minaxi (Downers Grove, IL, U.S.A.) or a Packard Mark III liquid scintillation spectrometer with Picofluor 30 (Packard) as a scintillation cocktail.

III EXPERIMENTAL PROCEDURES

III.1 Development of assays for analysis of sulfur mustard adducts to proteins as Standard Operating Procedures: mass spectrometric analysis of an alkylated tripeptide in albumin

III.1.1 Incubation of human blood with sulfur mustard, [^{14}C]sulfur mustard or sulfur mustard- d_8

A 1 M solution of sulfur mustard, [^{14}C]sulfur mustard (sp. act. 15 mCi/mmol) or sulfur mustard- d_8 in CH_3CN was prepared. Subsequently, the required dilution in CH_3CN was prepared and a well-defined amount was added to human blood (2-10 mL), resulting in a 1% end concentration of CH_3CN . After incubation for 2 h at 37 °C, plasma and erythrocytes were separated by centrifugation at 3,000 rpm.

III.1.2 Isolation of albumin from human plasma, using affinity chromatography

Plasma (1 mL) was applied on a HiTrapTM Blue HP (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) affinity column that was incorporated into an FPLC system, after conditioning with buffer A (50 mM KH_2PO_4 , pH 7; 10 mL). The column was eluted with buffer A (7 mL; 1 mL/min). A large peak was visible at 280 nm, corresponding with material having no affinity to the column material. Subsequently, the column was eluted with buffer B (50 mM KH_2PO_4 , 1.5 M KCl, pH 7; 7 mL; 1 mL/min). UV positive (280 nm) material was collected (total volume 2.5 mL). The HiTrap column was regenerated by washing with buffer A (14 mL).

Subsequently, a PD-10 column (containing 10 mL of Sephadex G 25 material) was equilibrated with 50 mM NH_4HCO_3 (25 mL). The albumin fraction, collected from the HiTrap Blue HP column (2.5 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50 mM; 0.5 mL). Next, the column was further eluted with aqueous NH_4HCO_3 (50 mM; 2.5 mL) and the eluate was collected.

III.1.3 Digestion of albumin obtained after affinity chromatography, with addition of internal standard

Plasma samples (1 mL), isolated from blood exposed to different concentrations of sulfur mustard were spiked with plasma (50 μL), isolated from blood exposed to 100 μM sulfur mustard- d_8 . Next, these samples were applied to a HiTrap Blue Sepharose column and desalted on a PD-10 column, as described above.

Pronase digestion of a sample (0.25 mL) of the purified albumin fraction (diluted with aqueous NH_4HCO_3 ; 50 mM; 0.5 mL), followed by LC/MS/MS analysis, showed a linear relationship between exposure level and peak ratio.

III.1.4 Tentative SOP for albumin – tripeptide assay

The plasma samples (1 mL) of interest were spiked with plasma (50 μL), isolated from blood exposed to 100 μM d_8 -sulfur mustard. The sample (1 mL) was applied on a HiTrapTM Blue HP (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) affinity column that was incorporated into an FPLC system, after conditioning with buffer A (50 mM KH_2PO_4 , pH 7; 10 mL). The column was eluted with buffer A (7 mL; 1 mL/min). A large peak was visible at 280 nm, corresponding with material having no affinity to the column material. Subsequently, the column was eluted with buffer B (50 mM KH_2PO_4 ,

1.5 M KCl, pH 7; 7 mL; 1 mL/min). UV positive (280 nm) material was collected (total volume 2.5 mL). The HiTrap column was regenerated by washing with buffer A (14 mL). Subsequently, a PD-10 column (containing 10 mL of Sephadex G 25 material) was equilibrated with 50 mM NH_4HCO_3 (25 mL). The albumin fraction, collected from the HiTrap Blue HP column (2.5 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50 mM; 0.5 mL). Next, the column was further eluted with aqueous NH_4HCO_3 (50 mM; 2.5 mL) and the eluate was collected.

Part of the purified albumin fraction (0.25 ml) was diluted with aqueous NH_4HCO_3 (50 mM; 0.5 mL) and subsequently Pronase was added (100 μl of a freshly prepared solution (10 mg/ml) in 50 mM NH_4HCO_3), followed by incubation for 2 h at 37 °C. The digests were filtrated through molecular weight cut-off filters (10 kD) under centrifugation at 2772 g in order to remove the enzyme. The filtrate was analyzed by means of LC/MS/MS.

Conditions LC-system

Eluent A: 0.2% formic acid in water. Eluent B: 0.2% formic acid in acetonitrile.

| Time (min) | % eluent A | % eluent B | Flow (ml/min) |
|------------|------------|------------|---------------|
| 0 | 100 | 0 | 0.1 |
| 5 | 100 | 0 | 0.6 |
| 50 | 30 | 70 | 0.6 |

The flow of 0.6 ml/min was split before the column to 35 $\mu\text{l}/\text{min}$. Column: PepMap C18, 3 μm , 15 cm x 1 mm. Loop: 50 μl .

Conditions triple quad MS

Transitions were monitored of the protonated molecular ions of (S-HETE)Cys-Pro-Phe and (S- d_8 -HETE)Cys-Pro-Phe to the most intense fragment (HETE):

$\text{MH}^+ 470.2 \rightarrow 105$

$\text{MH}^+ 478.2 \rightarrow 113$

Scan time 1.2 s. Cone voltage 35 V, collision energy 20 eV (Argon pressure 3×10^{-3} mBar).

III.1.5 Synthesis of (S-HETE)Cys-Pro-Tyr

This compound was synthesized on solid phase, in an analogous way as (S-HETE)Cys-Pro-Phe, using N-Fmoc-(S-HETE)Cys (11). Tandem mass spectrometric data: m/z 486.2 (MH^+), 469.2 ($\text{MH}^+ - \text{NH}_3$), 279.2 (Pro-Tyr), 137.0 ($^+\text{S-CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-OH}$), 105.0 ($^+\text{CH}_2\text{-CH}_2\text{-S-CH}_2\text{-CH}_2\text{-OH}$).

III.1.6 Determination of persistence of sulfur mustard adducts in rats exposed to sulfur mustard (i.v.)

Male Wistar WU rats (approximately 300 g) were purchased from Harlan, The Netherlands. The animals were allowed to eat and drink ad libitum. They were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. The protocols for animal experiments were approved by the TNO Committee on Animal Care and Use. Rats (three animals per time point) were exposed to a dose of 0.3 mg sulfur mustard/kg (i.v.). Sulfur mustard was diluted to a concentration of 6 mg/mL in 2-propanol. Just before injecting the rats, the sulfur mustard solution was diluted with saline to give a concentration of 0.3 mg/mL. After anesthesia with Dormicum/Hypnorm, two rats at a time were injected with this freshly prepared solution (1 mL/kg, i.v.) in the penis vein.

At the requisite time points (10 min, 1 h, 6 h, 1, 2, 3, 7, and 28 days) after exposure, animals were killed by decapitation, blood (ca. 7 ml/rat) was collected in heparinized tubes and centrifuged (2500 rpm, 5 min) to separate plasma from erythrocytes. The plasma samples were stored at -20°C until further work-up. The erythrocytes were washed three times (2500 rpm, 5 min) with PBS and were subsequently stored at -20°C.

III.1.7 Preparation of "internal standards" for analysis of albumin and hemoglobin adducts

Rat blood (10 mL, heparinized) was incubated with sulfur mustard- d_8 in acetonitril (100 μ M; final acetonitril concentration 1%) for 2 h at 37 °C. Next, plasma was separated from erythrocytes by centrifugation (2500 rpm, 5 min). The plasma was stored at -20 °C and was used as such as internal standard for analysis of the plasma samples generated throughout the animal experiments. The erythrocytes were washed three times (2500 rpm, 5 min) with PBS, and hemolyzed in water. Subsequently globin was isolated according to the procedure described by Bailey *et al.* (19) and used as internal standard for analysis of the globin samples generated throughout the animal experiments.

III.1.8 Work-up of rat plasma samples

To rat plasma (0.5 mL) was added plasma (25 μ L), isolated from rat blood, exposed to 100 μ M sulfur mustard- d_8 . This mixture was diluted with buffer A (2 mL, 50 mM KH_2PO_4 , pH 7.0) and filtrated using a 0.45 μ m filter disc in order to remove solid particles. Next, the sample was applied on a Hi Trap Blue Sepharose column (Amersham Biosciences, 1 mL, capacity 20 mg HSA/mL gel) and washed with buffer A (12 mL). Next, albumin was eluted using buffer B (50 mM KH_2PO_4 , 1.5 M KCl, pH 7.0). The entire wash and elution steps were monitored with a UV lamp at 280 nm. UV positive material was collected, resulting in a total volume of 3 mL. These 3 mL samples were desalted using a PD-10 desalting column (Amersham Biosciences). The PD-10 column was equilibrated using a solution of NH_4HCO_3 (50 mM, 25 mL). Next, the sample consisting of buffer B (3 mL) and albumin was applied. The albumin was eluted using 3 mL of NH_4HCO_3 solution (50 mM).

III.1.9 Pronase digestion of rat albumin, followed by LC/MS/MS analysis

Part of the above solution (0.75 mL, containing maximal 4.8 mg albumin) was digested using Pronase (100 μ L, 10 mg/mL in 50 mM NH_4HCO_3) for 2 h at 37 °C. After 2 h the mixture was filtrated using a 10 kD ultrafilter. The filtrate was analyzed using Q-TOF LC-MS and LC/MS/MS for the presence of (S-HETE)Cys-Pro-Tyr and its deuterated analogue.

Conditions LC-system

Eluent A: 0.2% formic acid in water. Eluent B: 0.2% formic acid in acetonitrile.

| Time (min) | % eluent A | % eluent B | Flow (ml/min) |
|------------|------------|------------|---------------|
| 0 | 100 | 0 | 0.1 |
| 5 | 100 | 0 | 0.6 |
| 50 | 30 | 70 | 0.6 |

The flow of 0.6 ml/min was split before the column to 35 μ l/min. Column: PepMap C18, 3 μ m, 15 cm x 1 mm. Loop: 50 μ l.

Conditions triple quad MS

Transitions were monitored of the protonated molecular ions of (S-HETE)Cys-Pro-Tyr and (S-*d*₈-HETE)Cys-Pro-Tyr to the most intense fragment (HETE):

MH⁺ 486.2 → 105

MH⁺ 494.2 → 113

Scan time 1.2 s. Cone voltage 35 V, collision energy 20 eV (Argon pressure 3 × 10⁻³ mBar).

III.1.10 Analysis of rat globin samples according to the Standard Operating Procedure for modified Edman degradation of globin

Globin (20 mg), isolated (19) from rat blood, was dissolved in formamide (2 ml). Subsequently, a solution of globin isolated from blood exposed to sulfur mustard-*d*₈ (100 μM) in formamide (20 mg/ml; 50 μl) was added. Next, pyridine (8 μl) and pentafluorophenyl isothiocyanate (8 μl) were added and the mixture was incubated at 60 °C in a heating block for 2 h. After cooling to room temperature, the mixture was extracted with toluene (3 × 1 ml) by means of mixing the toluene with the formamide solution using a Vortex (30 s) and centrifuging in a Jouan RC 10.10 centrifugal evaporator for 2 min (1200 rpm). Next, the samples were frozen in liquid nitrogen in order to achieve a better separation of the two layers. The toluene layers were combined, washed with water (2 × 0.5 ml), aqueous Na₂CO₃ (0.1 M, 0.5 ml) and water (0.5 ml). The organic layer was dried (MgSO₄), evaporated to dryness using the centrifugal evaporator and dissolved in toluene (100 μl).

Next, a Florisil cartridge was conditioned with methanol/dichloromethane (1/9, v/v; 2 ml) and dichloromethane (2 ml), respectively. The toluene solution was applied on the cartridge, which was subsequently washed with dichloromethane (2 ml) and methanol/dichloromethane (1/9, v/v; 1 ml). The thiohydantoin was eluted with methanol/dichloromethane (1/9, v/v; 1.5 ml). The latter eluate was evaporated to dryness and dissolved in toluene (100 μl). To this solution heptafluorobutylidene imidazole (10 μl) was added and the mixture was heated at 60 °C for 30 min. After cooling, the reaction mixture was washed with water (2 × 100 μl), aqueous Na₂CO₃ (0.1 M, 100 μl) and finally with water (100 μl). The toluene layer was dried (MgSO₄), concentrated to 30 μl and analyzed with GC-MS.

Conditions of the GC-MS were as follows:

Column: Restek 451548 Rtx-5Sil-MS, length 30 m, i.d. 0.25 mm, film thickness 1 μm. Carrier gas: helium. Oven temp.: 140 °C (1 min) – 20 °C/min → 260 (18 min). Inj. volume: 1 μl via autosampler. Inj. temp. 270 °C. Inj. Mode: pulsed splitless. Pulse pressure/time 200 kPa/0.2 min. Splitless time : 1 min.

MSD transferline: 240 °C. MS quad: 130 °C. MS source: 160 °C. EMV: 1576 V. MSD mode: SIM, m/z 564, m/z 572. Dwell time : 80 msec.

III.2 Development of assays for analysis of sulfur mustard adducts to proteins as Standard Operating Procedures: mass spectrometric and fluorescence-based analysis of histidine adducts

III.2.1 Fluorescence derivatization of histidine adducts

FAM derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine

To a solution of N1- or N3-(2-hydroxyethylthioethyl)histidine (1 mg) in aqueous NaHCO₃ (1 ml, 0.1 M, pH 8.3) was added a solution of 5- and 6-carboxyfluorescein succinimidyl ester in DMF (150 μl, 42 mM). The mixture was vigorously vortexed and left at room temperature under the exclusion of light. A reaction mixture without the histidine derivative was taken as control. After 5 h, HPLC analysis demonstrated the presence of a strong UV-absorbing

compound for each isomer, which was not present in the chromatogram obtained for the control sample. The compounds were purified by elution on Seppak C18.

Yield: 0.23 mg N3 isomer and 0.39 mg N1 isomer (based on $^1\text{H-NMR}$ spectroscopy).

Electrospray MS: m/z 309.6 (MH_2^{2+}), 618.1 (MH^+) for both isomers.

III.3 Development of immunoslotblot assays for quantitative analysis of sulfur mustard adducts to proteins

III.3.1 Exposure of human skin to sulfur mustard vapor

The device as represented in Figure 1 was used for exposure of human skin. The estimated sulfur mustard concentration at 28 °C close to the skin was $1100 \text{ mg}\cdot\text{m}^{-3}$.

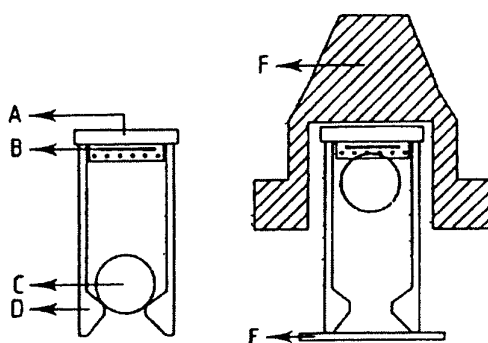


Figure 1. Schematic diagram of the device for skin exposure to air saturated with sulfur mustard vapor. At the inner side of the plastic cap (A) a piece of filter paper (B) was placed, onto which 3 μl of liquid sulfur mustard was applied. The bottom side of the glass cylinder was closed by a stainless steel ball (C). When the air in the cylinder (a volume of ca. 2 ml) had become saturated with sulfur mustard vapor (at 28 °C: ca. $1100 \text{ mg}/\text{m}^3$), the cylinder was placed onto the skin (E) and the ball was lifted with a magnet (F) for the desired period of time.

In addition, pieces of skin ($0.5 \times 0.5 \text{ cm}$) were covered with a solution of sulfur mustard (1 ml of 0, 50 or $100 \mu\text{M}$) in PBS containing 1% acetonitrile, for 30 min.

III.3.2 Preparation of skin cryostat sections

After exposure, a piece of the skin was cut from the central part of the treated area and fixed in methanol/acetic acid (3/1 v/v, 1.5 h at 4 °C), rehydrated by incubation overnight in 70% ethanol at 4 °C, followed by incubation in 5% sucrose at 4 °C for 1.5 h. Next, the pieces were stretched between microscope slides and stored at -20 °C.

Alternatively, the piece of skin was immediately stretched between microscope slides, without fixation, and stored at -20 °C.

For the preparation of cryostat sections, a small piece of skin was embedded in Tissue Tek (O.C.T. compound, Miles Inc., Elkhart, USA). Subsequently, cryostat sections (5 mm thickness) were prepared at -35 °C with a cryostat microtome (2800 Frigocut, Reichert-Jung, Leica, Rijswijk, The Netherlands) on slides precoated with a solution of 3-aminopropyl triethoxysilane (2% in acetone). The slides were stored at room temperature. In the case of non-fixed skin, the cross-sections were fixed with 70% ethanol, washed with TBS (20 mM Tris HCl, 150 mM NaCl, pH 7.4) and stored at room temperature.

III.3.3 Detection of keratin adducts; isolation of keratin from human callus

Human callus (100 mg) was soaked in Tris.HCl buffer (5 ml, 20 mM, pH 7.4) overnight. After centrifugation (30 min, 400 rpm) the residue was stirred in a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM) and urea (8 M). After centrifugation (30 min, 400 rpm), the residue was extracted with a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM), urea (8 M), and β -mercaptoethanol (0.1 M).

The crude keratin was purified on a G 75 column (100 \times 2 cm) with a buffer (pH 7.6) containing SDS (0.5%), Tris.HCl (10 mM) and DTT (10 mM); flow, 0.25 ml/min. Appropriate fractions were collected and dialysed against water. The remaining solution was lyophilized. Representative yield: 20 mg keratin/100 mg callus.

III.3.4 Exposure of human callus to sulfur mustard

To a suspension of human callus (70-100 mg) in 0.9% NaCl (100 μ l) was added a solution of an appropriate concentration of sulfur mustard in isopropanol (100 μ l). The mixture was incubated for 6 h at 37°C. Isolation of keratin was performed as described in Subsection III.3.3.

III.3.5 Exposure of human skin to saturated sulfur mustard vapor and extraction of epidermal keratins

Human skin samples (9 \times 0.25 cm²) were exposed to saturated sulfur mustard vapor (0, 2, 4 or 8 min) according to the method described in Subsection III.3.6. After the exposure, the epidermis was separated from the dermis by heat shock (2 min at 60 °C, followed by 5 min at 0 °C). The epidermis was cut into 3 or 4 pieces, and transferred into a 4 ml vial. Next, low salt buffer (10 mM Tris/150 mM NaCl/3 mM EDTA/0.1% N-P40; pH 7.4; 3 ml), containing a protease inhibitors cocktail (1 tablet/12 ml buffer), was added. The mixture was shaken for 1 h at 0 °C. The epidermis was sedimented, the upper layer was discarded and replaced by high salt buffer (10 mM Tris/150 mM NaCl/1.5 M KCl/3 mM EDTA/0.1% N-P40; pH 7.4; 3 ml) containing the protease inhibitors cocktail (1 tablet/12 ml buffer). The mixture was shaken for 1 h at 0 °C. Next, the epidermis was sedimented; the upper layer was discarded and replaced by washing buffer (10 mM Tris/150 mM NaCl/3 mM EDTA; pH 7.4; 3 ml) containing the protease inhibitors cocktail (1 tablet/12 ml buffer). After shaking for 30 min at 4 °C, the epidermis was sedimented. The liquid layer was discarded and the epidermis was extracted with lysis buffer (20 mM Tris/1 mM EDTA/2% SDS/1 mM DTT; pH 7.4, 2 ml), containing protease inhibitors, under gentle shaking overnight at room temperature. The mixture was centrifuged at 5,000g for 5 min and the supernatant containing the keratins (0.87 mg/ml) was stored at -70 °C.

III.3.6 Preparation of skin cryostat section and immunofluorescence microscopy

The preparation of skin cryostat sections has been described in Subsection III.3.2. Immunofluorescence microscopy of sulfur mustard-keratin adducts in skin sections was performed analogously to the procedure described for detection of N7-HETE-Gua (12). Briefly, the following procedure was applied after fixation of the skin section with 70% ethanol on aminoalkylsilane-precoated slides and washing with TBS:

- precoating with TBS + 5% milkpowder (30 min at room temperature);
- treatment with antibody specific for sulfur mustard-exposed keratin; supernatants of up to 32 selected monoclonal antibodies in a 1:1 dilution in TBS containing 0.05% Tween 20 and 0.5% gelatin (overnight at 4 °C);

- treatment with a second antibody, FITC-labeled 'goat-anti-mouse', 75-fold diluted in TBS containing 0.05% Tween 20 and 0.5% gelatin (2 h at 37 °C);
- counterstaining with propidium iodide (100 ng/ml, 10 min at room temperature).

Twin images were obtained with a LSM-41 laser scanning microscope. The fluorescence of the FITC group above the stratum corneum and of the propidium iodide were measured consecutively to visualize the presence of sulfur mustard-keratin adducts in the stratum corneum and the DNA in the nuclei. The fluorescein staining was used to determine the presence of sulfur mustard-keratin. Adduct levels were estimated from the brightness of the fluorescence above the stratum corneum. The second image, from the propidium iodide staining, served to localize nuclei on the image.

III.3.7 Immunoassays (ELISA) with hybridoma-supernatants

As described under 'Results' the screening of hybridoma supernatants was subject to detailed investigation. So far the following procedures yielded optimal results:

The hybridoma-supernatants were tested in a direct ELISA against globin isolated from human blood treated with sulfur mustard (0, 50, 100, 500 μ M). The ELISA was performed as follows. Polystyrene 'high binding' 96-well microtiter plates were coated with adducted and non-adducted globin dissolved in PBS to a final concentration of 10-12.5 μ g/ml. Of these dilutions 50 μ l was added per well and incubated 1 h at room temperature. The plates were washed three times with PBS. Next, the plates were incubated with PBS containing 2% FCS for 30 min at room temperature. After emptying the plates, the hybridoma supernatants were added, diluted 100-500 times in PBS with 0.05% Tween 20 and 1% FCS. Of these dilutions 50 μ l was added per well and incubated for 30 min at room temperature. After washing with PBS containing 0.05% Tween 20 (three times), the second antibody, viz., goat-anti-mouse-Ig(total)-alkaline phosphatase diluted 500-2000 times in PBS containing 0.05% Tween 20, and 1% FCS, was added (50 μ l/well) and the plates were incubated for 60 min at room temperature. After four washings with PBS containing 0.05% Tween 20, a solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$; 50 μ l) was added as a substrate for alkaline phosphatase and the mixture was incubated at 37 °C for 30 min.

For the screening of supernatants in a direct ELISA against sulfur mustard adducts to keratin the same procedure was applied, with the following modifications. The plates were coated with keratin isolated from human callus treated with sulfur mustard (0 or 50 μ M), dissolved in PBS to a final concentration of 5 μ g/ml. After washing with PBS, the plates were incubated with PBS containing 2% FCS, followed by washing with PBS containing 0.05% Tween 20 (three times). Hybridoma supernatants were screened in a 1:5 dilution. Further processing was the same as described above for plates coated with globin.

III.3.8 Immunoblot procedure for the detection of sulfur mustard adducts to keratin

Essentially, the procedure developed for the detection of sulfur mustard adducts to DNA (12) was applied. Briefly, a solution of keratin (200 μ l, 2.5 μ g/ml PBS) isolated from a human callus sample exposed to sulfur mustard was spotted on a nitrocellulose filter. All samples were blotted in triplicate on the same filter. After blotting, the slots were rinsed with PBS. The filters were dried on air and the keratin was immobilized by UV-crosslinking (50 mJ/cm²). Next, the filter was incubated with blocking solution (about 50 ml, 5% milkpowder in PBS + 0.1% Tween 20) at room temperature for 30 min and washed three times with PBS + 0.1% Tween 20. Next, the filter was incubated with 1st antibody (1H10, directed against sulfur mustard adducts to human keratin) diluted 500-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, overnight at 4 °C under continuous shaking, washed 4 times with PBS + 0.1% Tween 20, the last three times for at least 15 min each. Next, the filter was incubated with 2nd antibody (directed against the 1st antibody) diluted 1000-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, for 2 h at room temperature.

under continuous shaking, washed 4 times with PBS + 0.1% Tween 20, the last three times for at least 15 min each.

Finally, a 1:100 mixture of solution B with solution A (of the Enhanced Chemiluminescence Blotting Detection System) was preincubated in a water bath for at least 30 min at 25 °C. Then, free (wash) solution was removed from the filter with filter paper and position A12 and H1 marked with ball point (not a felt pen!). The filter was placed in a closely fitting box. After addition of substrate solution (10 ml) incubation was performed for 1 min.

Next, the filter was wrapped in plastic (straight from the substrate solution) without air bubbles. Liquid was pressed out, the filter was transferred in plastic into a luminometer cassette and placed in the luminometer. Luminescence was measured according to the required program. For each sample the mean luminescence of triplicate samples was calculated.

IV RESULTS

IV.1 Further development of the mass spectrometric analysis of the tripeptide (S-HETE)-Cys-Pro-Phe, resulting from pronase digestion of albumin alkylated by sulfur mustard (*Part A, t.o. 1-9*)

IV.1.1 Introduction

Results within the framework of our previous grant DAMD17-97-2-7002 showed that pronase digestion of albumin alkylated by sulfur mustard resulted in the formation of the tripeptide (S-HETE)-Cys-Pro-Phe, which could be conveniently isolated and determined in a rather sensitive way by micro-LC/electrospray tandem MS with multiple reaction monitoring at an absolute detection limit of 4 pg (11). Most important results obtained in the first year of the current cooperative agreement showed that:

- the procedure for isolation of albumin from blood could be substantially shortened by using affinity chromatography (20; t.o. 1)
- the lowest detectable exposure level was improved by one of magnitude (1 nM) by working up of 20 mg amounts of albumin (t.o. 3)

Presently, this is by far the most sensitive method for detection of exposure of human blood to sulfur mustard. Interestingly, we recently showed that this method can also be applied to demonstrate exposure (*in vitro* as well as *in vivo*) to a wide range of nitrogen mustard derivatives (14).

In the second year of the grant period the method was further developed into a tentative Standard Operating Procedure.

IV.1.2 Use of albumin alkylated by sulfur mustard- d_8 as an internal standard (*t.o. 2*)

The use of an internal standard, *i.e.*, albumin isolated from human blood exposed to sulfur mustard- d_8 , has now been completely worked out. This will enable quantitative analyses of unknown samples. See Figure 2 for representative ion chromatograms for analysis of (S-HETE)Cys-Pro-Phe and (S- d_8 -HETE)Cys-Pro-Phe in a Pronase digest of albumin, and Figure 3 for the corresponding MS/MS spectra. The procedure will be used in subsequent experiments, *e.g.*, the animal experiments.

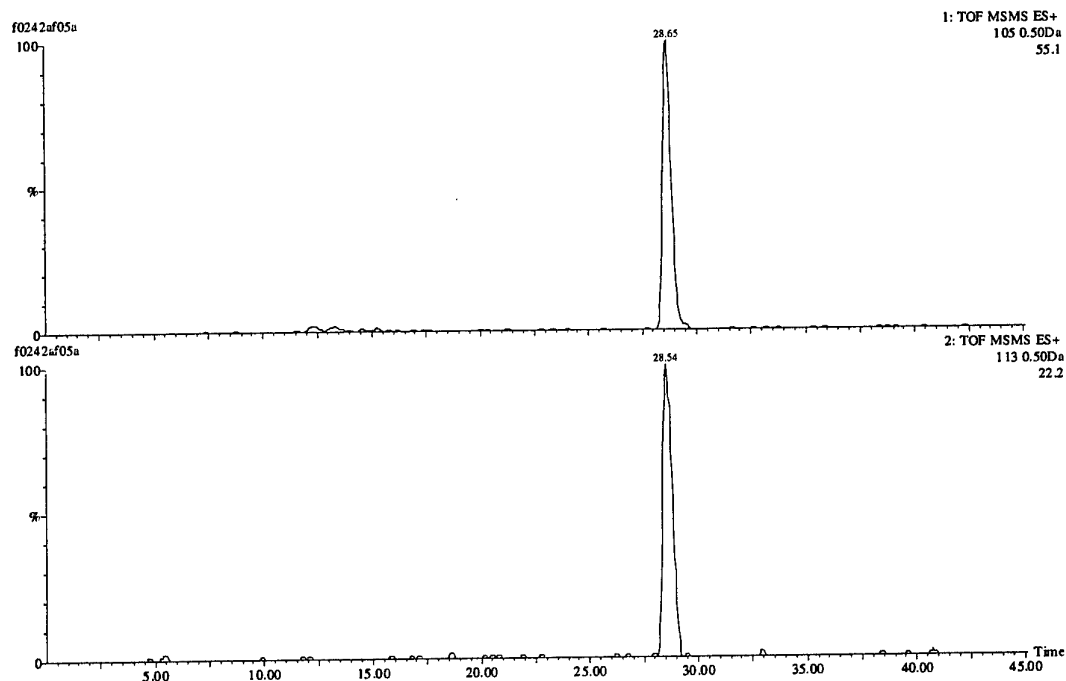


Figure 2. LC-tandem MS analysis (Q-TOF instrument) of (S-HETE)Cys-Pro-Phe (upper panel) and (S-*d*₈-HETE)Cys-Pro-Phe (lower panel) in a pronase digest of albumin. Albumin was isolated from plasma (1 ml) by affinity chromatography, followed by desalting on a PD-10 column. The plasma was isolated from human blood that had been exposed to sulfur mustard (10 μ M). Prior to isolation of albumin, 50 μ l of a plasma sample was added, isolated from blood that had been exposed to sulfur mustard-*d*₈ (100 μ M).

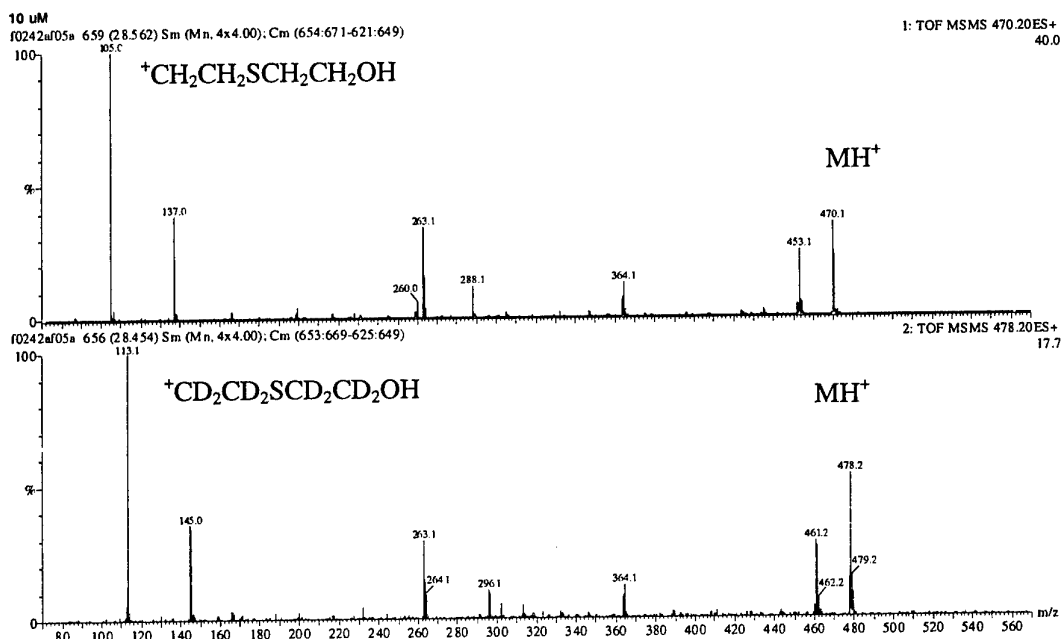


Figure 3. Tandem MS spectra (Q-TOF instrument) of (S-HETE)Cys-Pro-Phe (upper panel) and (S-*d*₈-HETE)Cys-Pro-Phe (lower panel).

IV.1.3 Persistence of albumin adducts (*t.o.* 8 and 9)

In order to obtain information about the persistence of the albumin – sulfur mustard adduct, laboratory animals were exposed to the agent. We prefer to use the rat for these studies because the amino acid sequence of rat albumin has been published². Furthermore, it has been reported that the modified tripeptide Cys-Pro-Tyr results from rat albumin modified at Cys-34 by the food-borne carcinogens IQ (21) and PhIP (22), after pronase treatment. Preliminary experiments (conducted in the first year of this cooperative agreement), in which rat blood had been exposed *in vitro* to sulfur mustard, showed that the major product was (S-HETE)Cys-Pro-Tyr (see Figure 4 for tandem MS spectrum), and that the level of alkylation of the free cysteine residue in rat albumin was approximately 5%. Electrospray tandem mass spectrometric analyses could be performed in an analogous way, *i.e.*, by selecting the charged molecular ion in the first MS and measuring the highly selective 105 fragment in the second mass spectrometer. Consequently, the rat was chosen as laboratory animal for studying the persistence of the albumin adduct.

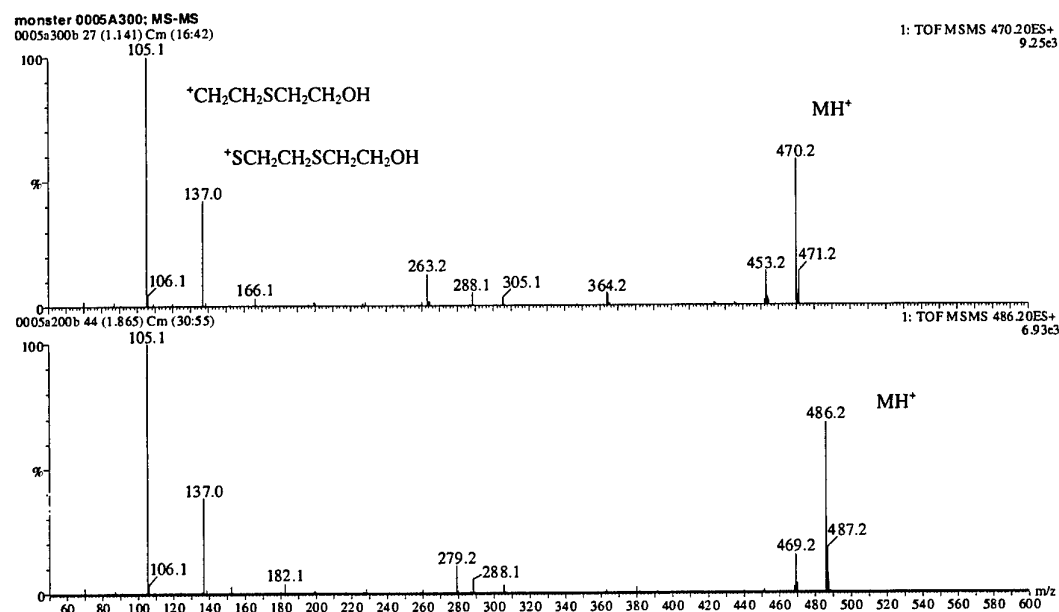


Figure 4. Comparison of tandem MS spectra (recorded on Q-TOF instrument) of (S-HETE)Cys-Pro-Phe (upper panel) and (S-HETE)Cys-Pro-Tyr (lower panel), demonstrating the intense fragment ion with m/z 105 in both cases.

² The amino acid sequence of guinea pig albumin is unknown. In an experiment in which guinea pig albumin (isolated from sulfur mustard-exposed guinea pig blood) was digested with pronase, no tripeptide adduct could be detected.

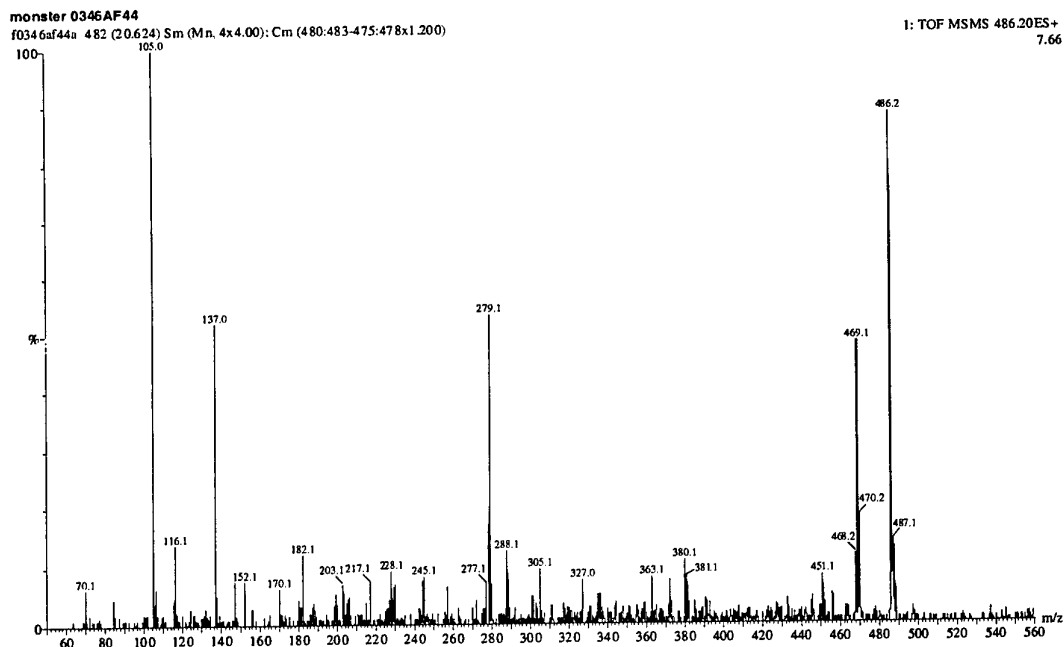


Figure 5. Full scan electrospray tandem MS spectrum (recorded on Q-TOF instrument) of molecular ion (MH^+ 486.2) of sulfur mustard tripeptide adduct (S-HETE)Cys-Pro-Tyr, obtained after pronase digestion of albumin from rat blood, isolated 1 h after exposure *in vivo* to sulfur mustard (0.3 mg/kg, i.v.).

The animal study was performed as follows. For each time point to be studied 3 rats were taken. A dose of 0.3 mg/kg sulfur mustard was administrated (i.v.) to the animals, and blood was collected after 10 min, 1 h, 6 h, 1, 2, 3, 7 and 28 days after exposure. At the indicated time points, blood was collected (approximately 7 mL per rat). Plasma was separated from the erythrocytes by centrifugation and after addition of well-defined amount of internal standard (i.e., plasma from rat blood that had been exposed to 100 μ M sulfur mustard- d_8) albumin was isolated by affinity chromatography on Blue Sepharose cartridges, according to the tentative SOP. After treatment with Pronase, the resulting digests were analyzed qualitatively by means of LC tandem MS-MS on a Q-TOF instrument, with recording of full MS-MS spectra, in order to verify that indeed the tripeptide (S-HETE)Cys-Pro-Tyr was formed *in vivo*; see Figure 5 for a full MS-MS spectrum.

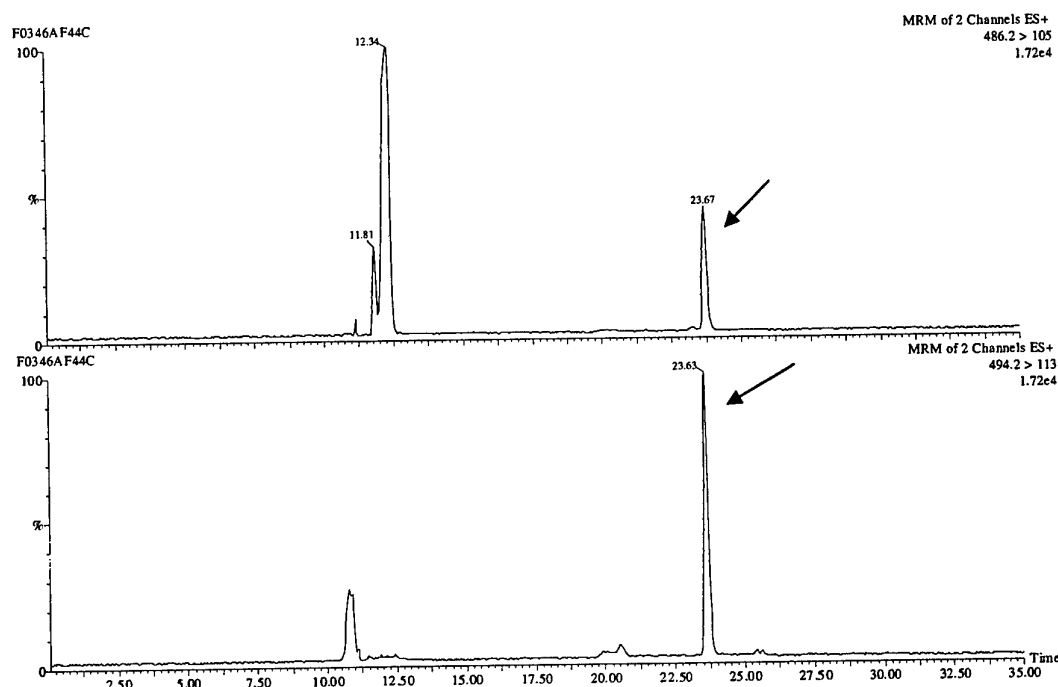


Figure 6. LC-tandem MS analysis of (S-HETE)Cys-Pro-Tyr (arrow) in a pronase digest of albumin, using the multiple reaction monitoring scanning mode for the transition m/z 486 (MH^+) \rightarrow 105. Albumin was isolated from plasma taken from a rat, 1 h after exposure to sulfur mustard (0.3 mg/kg). The sample was analyzed in the presence of albumin, isolated from rat blood exposed to sulfur mustard- d_8 *in vitro* (corresponding to 5 μ M sulfur mustard- d_8). Upper panel: analysis of (S-HETE)Cys-Pro-Tyr (multiple reaction monitoring of the transition m/z 486 (MH^+) \rightarrow 105). Lower panel: analysis of (S- d_8 -HETE)Cys-Pro-Tyr (multiple reaction monitoring of the transition m/z 494 (MH^+) \rightarrow 113)

Subsequently, the samples were analyzed by means of LC tandem MS on a triple-quad instrument for more sensitive analysis by using the multiple reaction monitoring mode. A representative analytical run is given in Figure 6. Significant amounts of the tripeptide could be observed, which rapidly decreased in time (half-life of sulfur mustard - modified albumin: 2 days). For the time-course of the albumin adduct level see Table 1 and Figure 7.

Table 1. Peak ratios of d_0/d_8 (S-HETE)Cys-Pro-Tyr levels after pronase digestion of albumin samples of rats after exposure to sulfur mustard (0.3 mg/kg, i.v.)

| sample | d_0/d_8 (1 st series) | d_0/d_8 (2 nd series) | d_0/d_8 (3 rd series) | d_0/d_8 (mean) |
|--------|------------------------------------|------------------------------------|------------------------------------|------------------|
| 10 min | 0.29 | 0.22 | * | 0.25 |
| 1 h | 0.46 | 0.42 | 0.32 | 0.40 |
| 6 h | 0.37 | 0.34 | 0.34 | 0.35 |
| 1 d | 0.20 | 0.16 | 0.20 | 0.18 |
| 2 d | 0.16 | 0.17 | * | 0.16 |
| 3 d | 0.16 | 0.10 | 0.16 | 0.14 |
| 7 d | 0.033 | 0.032 | 0.039 | 0.035 |
| 28 d | 0** | 0** | 0** | 0** |

* animal died just after administration of sulfur mustard

** the level of d_0 -(S-HETE)Cys-Pro-Tyr was below the detection limit

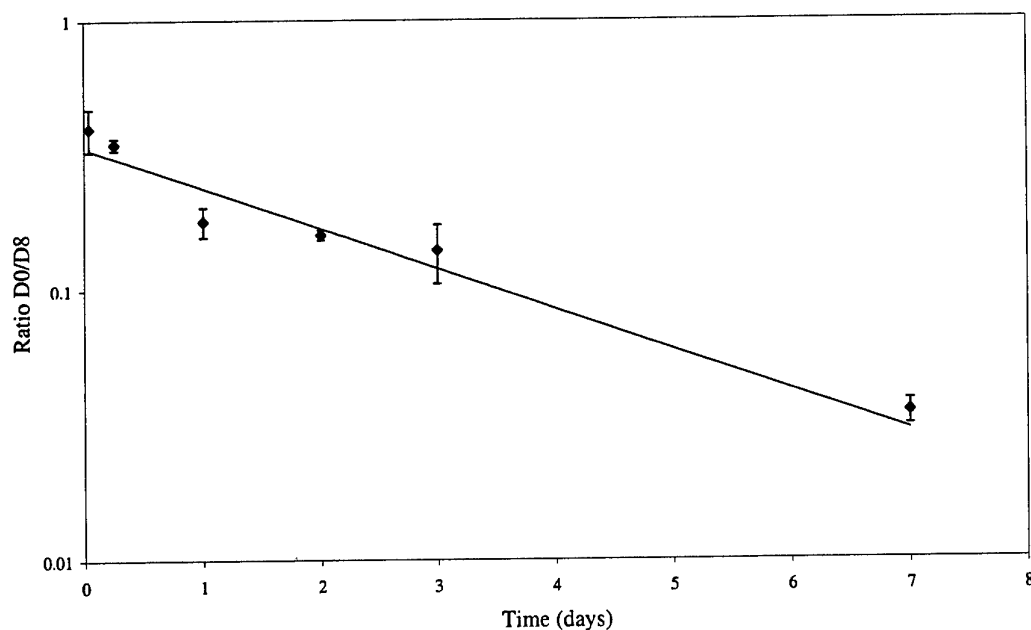


Figure 7. Persistence of alkylated cysteine in albumin of rats (n=3) after administration of sulfur mustard (0.3 mg/kg, i.v.). At the time points indicated blood samples were collected, albumin was isolated by affinity chromatography and analyzed by using the tentative SOP for determination of (S-HETE)Cys-Pro-Tyr. Plasma from rat blood exposed to sulfur mustard- d_8 (100 μ M) was used as an internal standard.

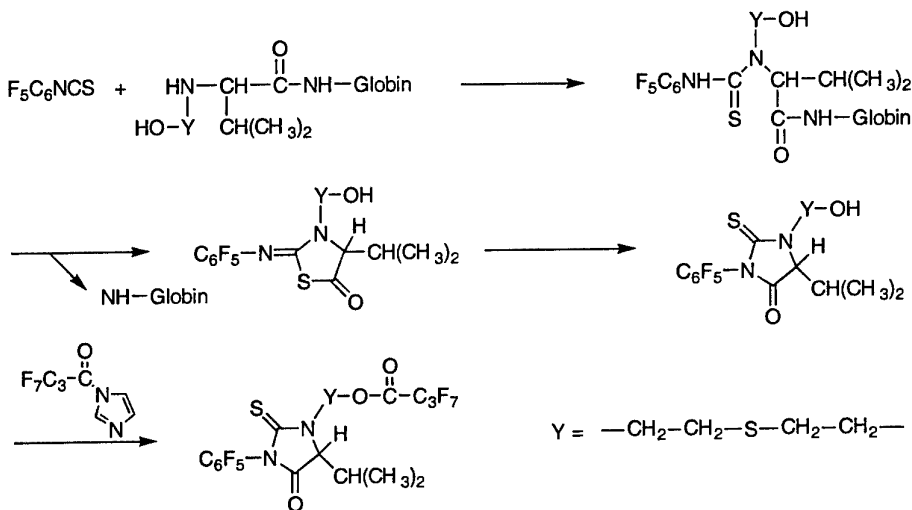


Figure 8. Modified Edman degradation of globin alkylated by sulfur mustard.

Subsequently, the N-terminal valine adduct levels were determined in the corresponding erythrocyte samples according to the Standard Operating Procedure (SOP) reported during the previous Cooperative Agreement DAMD17-97-2-7002. Globin was isolated and subsequently a well-defined amount of globin isolated from blood that had been exposed sulfur mustard- d_8 was added, and the mixture was processed according to the SOP. Thus, shortly, the globin samples were dissolved in formamide and pentafluorophenylisothiocyanate was added. After incubation for 2 h at 60 °C, the pentafluorophenylthiohydantoin was isolated by extraction and was further derivatized by treatment with heptafluorobutyrylimidazole (see Figure 8). The samples were analyzed by means of GC-NCI-MS with single ion monitoring for the presence of d_0 - and d_8 -pentafluorophenylthiohydantoin. A representative analytical run is shown in Figure 9. The results of the various analyses are given in Table 2 and Figure 10.

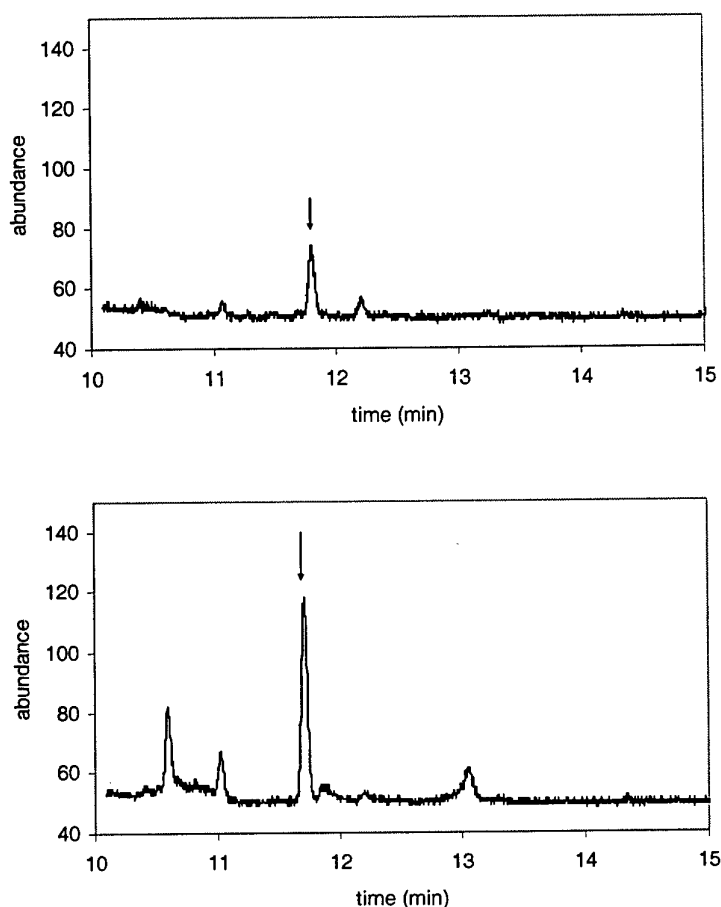


Figure 9. Representative GC-NCI/MS analysis of globin (20 mg) isolated from blood taken from a rat, 7 days after administration of sulfur mustard (0.3 mg/kg, i.v.), after modified Edman degradation. Globin isolated from rat blood treated with 100 μ M sulfur mustard- d_8 served as an internal standard. Ion chromatograms after monitoring for m/z 564 (analyte; upper panel) and m/z 572 (internal standard; lower panel).

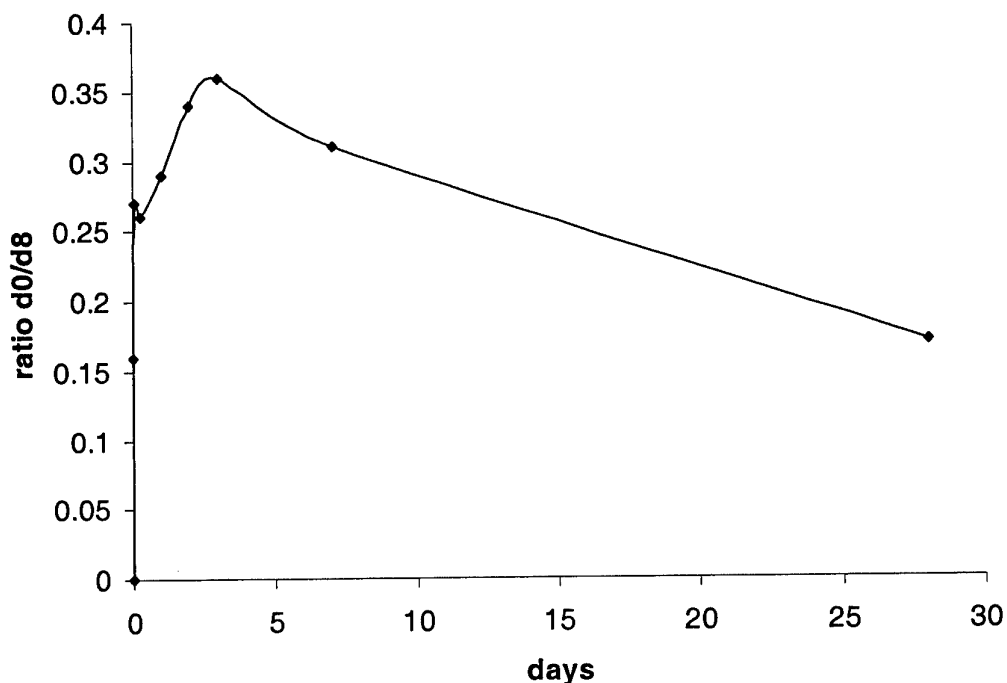


Figure 10. Persistence of alkylated N-terminal valine in hemoglobin of rats (n=3) after administration of sulfur mustard (0.3 mg/kg, i.v.). At the time points indicated blood samples were collected, globin was isolated and analyzed by using the SOP for determination of alkylated N-terminal valine. Globin from rat blood exposed to sulfur mustard- d_8 (100 μ M) was used as an internal standard.

Table 2. Peak ratios of d_0/d_8 pentafluorophenylthiohydantoin derivatives after modified Edman degradation of globin samples of rats after exposure to sulfur mustard (0.3 mg/kg, i.v.)

| sample | d_0/d_8 (1 st series) | d_0/d_8 (2 nd series) | d_0/d_8 (3 rd series) | d_0/d_8 (mean) |
|--------|------------------------------------|------------------------------------|------------------------------------|------------------|
| 10 min | 0.15 | 0.17 | * | 0.16 |
| 1 h | 0.37 | 0.24 | 0.19 | 0.27 |
| 6 h | 0.29 | 0.25 | 0.24 | 0.26 |
| 1 d | 0.24 | 0.29 | 0.34 | 0.29 |
| 2 d | 0.33 | 0.36 | * | 0.34 |
| 3 d | 0.40 | 0.31 | 0.38 | 0.36 |
| 7 d | 0.32 | 0.32 | 0.30 | 0.31 |
| 28 d | 0.18 | 0.16 | 0.17 | 0.17 |

* animal died just after administration of sulfur mustard

The results clearly demonstrate that the adduct level increases during the first days after the exposure. This implicates that there is still intact sulfur mustard present in the animal. Comparison of the two time-courses, i.e., from hemoglobin adduct and albumin adduct levels, learns that in the rat the hemoglobin adduct is far more persistent than the albumin adduct, as should be expected in view of the life time of the rat erythrocyte and the half life of rat albumin.

IV.2 Further development of methods for sensitive analysis of N1/N3-(2-hydroxyethylthioethyl)histidine (*part B, t.o. 10-13*)

IV.2.1 Introduction

In hemoglobin and albumin from human blood exposed to sulfur mustard, 34% and 28% of total adduct level could be ascribed to N1/N3-HETE-histidine, respectively (9). In view of this abundance and the stability of the adduct under acidic conditions, which enables its quantitative release from proteins, we investigated the mass spectrometric analysis of this adduct for which no sensitive method is currently available. The only published method is based on LC/electrospray tandem MS analysis of the 9-fluorenylmethoxycarbonyl (Fmoc) derivative of N1/N3-HETE-histidine, which allowed the determination of an exposure level of human blood *in vitro* of 10 μ M sulfur mustard from the adducts formed in hemoglobin (9).

In the first year of the cooperative agreement, the most relevant results were:

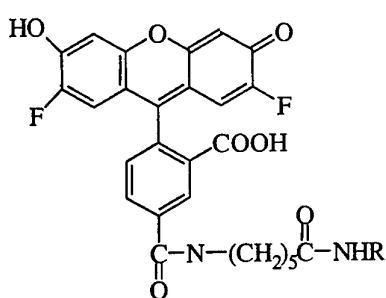
- a work-up procedure was developed for rapid isolation of the histidine adducts from amino acid mixtures resulting from acidic hydrolysis of protein material (*t.o. 10*)
- derivatization of the histidine adducts with MeOH/HCl and trifluoroacetic acid anhydride for GC-MS analysis resulted in a derivative with favorable mass spectrometric properties (*t.o. 11*)
- for detection with laser-induced fluorescence, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared (*t.o. 12*).

In the second year of the cooperative agreement, further attention was paid to *t.o. 12*.

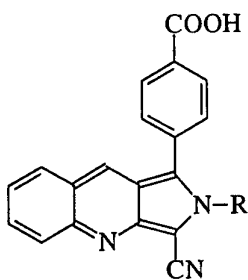
IV.2.2 Derivatization of N1/N3-HETE-histidine for HPLC with laser-induced fluorescence detection

Laser-induced fluorescence has been used for detection of yoctomole amounts of amino acid derivatives, whereas detection at single molecule level has been shown, albeit under extremely controlled conditions (23). This technique, however, is less selective than mass spectrometry. Various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared in the first year of the cooperative agreement (see Figure 11). In the second year of the agreement, the attempts to prepare promising fluorescent derivatives were continued.

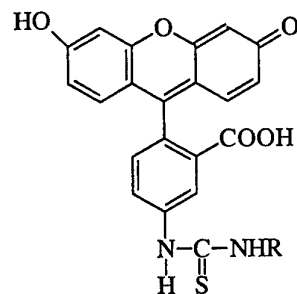
In our hands the most promising fluorescein-based reagent was 5/6-carboxyfluorescein (FAM) succinimidyl ester. The derivatization reaction proceeded smoothly and the stability of the carboxamide derivatives was excellent. The individual derivatives of both N1-HETE-His and N3-HETE-His were isolated by semi-preparative FPLC, using a reversed-phase column, and characterized with electrospray MS. The UV spectrum of one of the derivatives is given in Figure 12. The derivatives were stored as dry solids and proved stable for several months. With the fluorescent derivatives in hand, the detection limits were determined for analysis with capillary electrophoresis with laser induced fluorescence (CE-LIF) detection. See Figure 13 for a representative analysis of the reference compound. The detection limits of the FAM N1-HETE- and FAM N3-HETE-histidine derivatives were 24 and 21 pg/ml, respectively. The derivatization of amino acid mixtures (containing the histidine adducts) will be studied in more detail in the third year of the grant period.



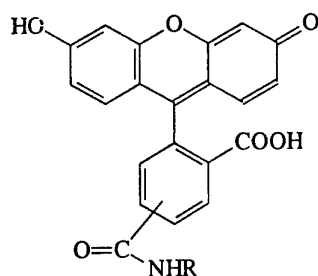
Oregon Green derivative



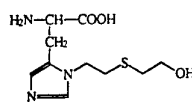
CBQCA derivative



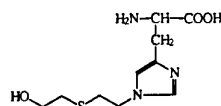
FITC derivative



FAM derivative



N1-isomer



N3-isomer

R = N1/N3-(2-hydroxyethylthioethyl)histidine

Figure 11. Fluorescent derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine.

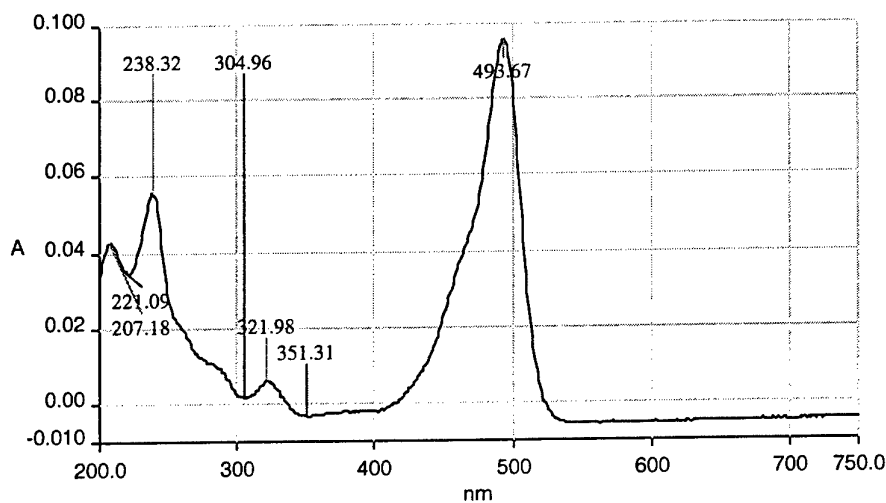


Figure 12. UV spectrum of FAM derivative of N1-HETE-histidine.

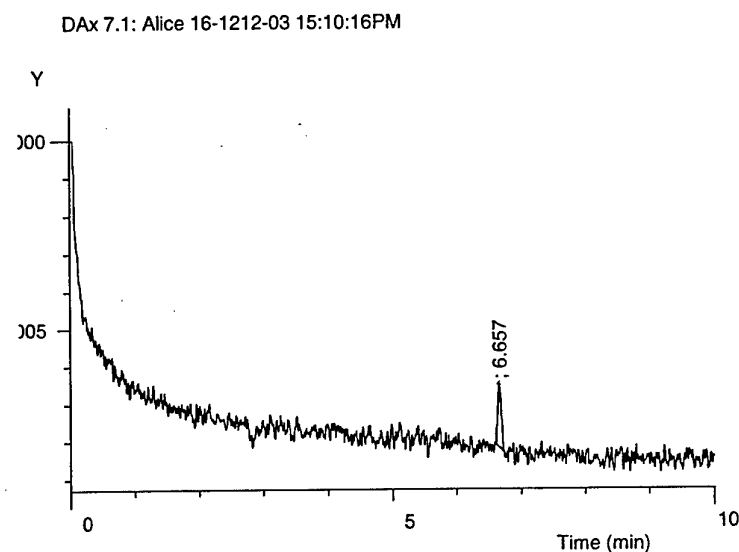
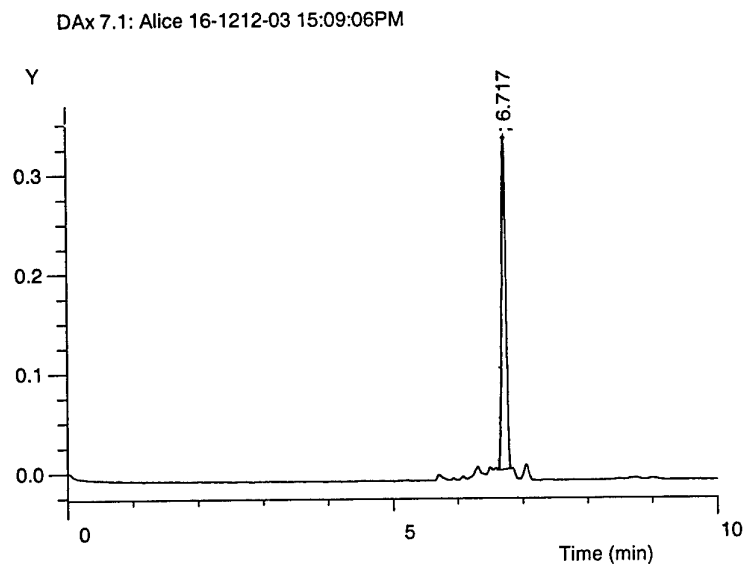


Figure 13. CE-LIF analysis of FAM derivative of N1-HETE-histidine at two different concentrations: 4.9 ng/ml (upper panel) and 24 pg/ml (lower panel).

IV.3 Optimization of immunoassay for screening of available antibodies against sulfur mustard adducts to proteins

IV.3.1 Introduction

The following steps were taken in our general approach to the development of an immunochemical assay for the detection of sulfur mustard adducts with proteins. It has been attempted to further improve the sensitivity and reproducibility of the ELISA for the screening of monoclonal antibodies against sulfur mustard adducts to hemoglobin, albumin and keratin. Next, some of the most promising clones were further characterized and applied in the development of the generally more sensitive immunoslotblot assay.

IV.3.2. Screening of monoclonal antibodies directed against sulfur mustard adducts to hemoglobin and globin in a direct ELISA

An ELISA assay was set up for the screening of antibodies against sulfur mustard adducts to hemoglobin. As described in the previous annual report several coating conditions for hemoglobin and globin were attempted, as well as variation of blocking of free places with water + 5% FCS or PBS + 2% FCS and variation of time and temperature of treatment with 1st ab and 2nd ab.

This resulted in the following procedure:

1. Coating of plates with globin (50 µl of 12.5 µg/ml, isolated from human blood exposed to 0 and 50 µM sulfur mustard) in PBS for 1 h at room temperature (or overnight),
2. washing with PBS (3 times),
3. blocking of free places with 2% FCS in PBS (30 min at room temperature),
4. washing with PBS + 0.05% Tween (3 times),
5. adsorption of 1st ab (in duplicates): supernatants of clone 183 3D5-2E11s obtained from sulfur mustard-N-terminal val-peptides fusion, and of 3H6, obtained from a mouse immunized with an alkylated peptide of hemoglobin (4), were applied in a 1:5 dilution, 30 min at room temperature,
6. washing with PBS + 0.05% Tween (3 times),
7. adsorption of 2nd ab: 1/1000 diluted GAM-alkaline phosphatase in PBS+0.05% Tween+1% FCS, 30 min at room temperature,
8. washing with PBS + 0.05% Tween (3 times), and with DEA (1 time),
9. incubation with MUP in reaction buffer, pH 9.8, 45 min at 37 °C,
10. measurement of fluorescence with microtiter plate reader.

The data with this procedure suggest that the immuno assay for screening of monoclonals producing ab's against sulfur mustard adducts to globin can still further be improved by suppressing the a-specific binding of 1st ab to globin, for example by carrying out the 1st ab adsorption at a higher concentration of Tween 20. When this results in a more sensitive and more reproducible assay than, after preparing fresh supernatant of each clone, all other available monoclonal clones can be screened for efficiency of ab- binding.

Due to the problems with the antibodies raised against sulfur mustard keratin adducts (see section IV.3.4) these experiments were postponed to the third year of the grant.

IV.3.3 Screening of monoclonal antibodies directed against sulfur mustard adducts to albumin in a direct ELISA

During the past period no supernatants of clones have been screened obtained from immunizations with alkylated peptides of human serum albumin

IV.3.4 Screening of monoclonal antibodies directed against sulfur mustard adducts to keratin in a direct ELISA

An ELISA assay was set up for the screening of antibodies against sulfur mustard adducts to keratin. More or less the same procedure was applied as originally applied for the screening of anti-sulfur mustard-globin clones, described in section IV.2.1., except that coating of plates

was carried out with keratin (50 μ l of 5 μ g/ml, isolated from human callus exposed to 0 and 50 μ M sulfur mustard) in PBS for 1 h at room temperature.

Several clones could be selected with a high specificity for keratin exposed to sulfur mustard. A number of these are presented in Table 3.

Other supernatants appeared to have lost their activity. For that reason 28 clones, all originating from the same clone 1.3-C2 from the mouse immunized with peptide 1 + 2, were cultured again to produce fresh supernatant. Although there was some differentiation in growing rate of the cell cultures, all supernatants appeared to be active, indicating that all clones were still able to produce antibodies specific for sulfur mustard adducts to keratin.

In an attempt to reproduce the screening results with antibodies specific for alkylated keratin, some problems arose. On keratin samples newly prepared from callus (obtained from other local chiropodists) exposed to sulfur mustard no specificity was observed. On the originally prepared keratin samples, all clones tested were still positive. Because this suggested that something was wrong with the isolation of the keratin samples several isolation procedures, described in literature were applied, but all without the wanted specificity for adducts of sulfur mustard to the keratin.

Briefly, we applied besides the originally applied method (24) a modification of this method by homogenizing the suspension (10 min), followed by sonification during the extraction phase. Also the method of Steinert (25) was applied in which extraction occurred in Tris (20 mM), urea (8 M) and β -mercaptoethanol (0.1 M). Upon extraction all preparations were centrifuged (30 min 3500 rpm) and the supernatants dialysed against water. Because dialysis resulted in the formation of a precipitate this step was substituted by filtration through Centrex UF-2 filters (molecular cut-off 10 kDa) followed by lyophilization.

Further studies on the protein samples indicated that assessment of the keratin concentrations in the samples, pipetted from the filters after three washings with water, resulted in erroneous results when the BioRad method was applied. This suggests that in spite of the repeated washings the keratin samples still contained compounds which interfere with the protein measurements. In addition, when applying the more reliable protein precipitation method a large variation in protein concentration (from 0.2 up to 3 mg/ml) was obtained. Both phenomena may have a confounding effect on the responses obtained in the direct ELISA.

In the current studies variations are applied in both the exposure conditions of human callus to sulfur mustard and the isolation procedure of keratin to improve the reproducibility of the response of sulfur mustard exposed keratin in the direct ELISA. In addition, the originally obtained primary clones which produced antibodies specific for sulfur mustard adducts to keratin will be subcloned, followed by selection in a direct ELISA with a coating of keratin isolated from newly sulfur mustard exposed human callus or keratin treated in solution with sulfur mustard.

A complicating factor appeared to be the fact that contamination of the isolated keratins with small amounts of SDS (essential for extraction of keratin) interfered with the binding of these keratins in the immunoassays which might result in erroneous outcomes of the immunoassays.

IV.4 Cross reactivity of antibodies for sulfur mustard adducts with hemoglobin, albumin and keratin.

Clone 3H6, which had been derived from a fusion with an adducted cysteine of a peptide derived from hemoglobin, showed some specificity for both alkylated keratin (Table 3)³.

The same clone, and some clones derived from fusions with an adducted his-peptide derived from hemoglobin did not show any specificity for alkylated human serum albumin. However, when applied on microtiter plates coated with alkylated keratin some clones derived from fusions with an adducted his-peptide derived from hemoglobin, showed some binding (Table 4; see also section IV.2.1). This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

Table 3. Antibody specificities of clones obtained from a fusion after immunization with haptens of partial sequences of keratin containing glutamine (1,2) or asparagin (3)-sulfur mustard adduct. Supernatants of cultures in a 1:5 dilution were assayed in a direct ELISA on keratin from human callus treated with 0 μ M or 50 μ M sulfur mustard. Fluorescence (in arbitrary units) is presented as a measure for antibody binding.

| clone | peptides used for immunization | Antibody response against keratin exposed to sulfur mustard solution of | |
|-----------|--------------------------------|---|------------|
| | | 0 μ M | 50 μ M |
| 2.3D7 | 3 | 124 | 537 |
| 3.2G8 | 1+2+3 | 294 | 1165 |
| 1.1A8 | | 2969 | 4013 |
| 1.2B6 | 1+2 | 275 | 1445 |
| 2.3D9 | 3 | 337 | 2709 |
| 2.2B5 | 3 | 494 | 3380 |
| 1.3C2-1D9 | 1+2 | 810 | 3423 |
| 1.3C2-1B4 | 1+2 | 641 | 3766 |
| 1H10 | 1+2 | 975 | 3791 |
| 1H10(2) | 1+2 | 618 | 3745 |
| 3H6 | | 586 | 1268 |

³ The complication with small amounts of SDS present in the keratin preparations might be of influence on the presented data.

Table 4. Antibody specificities of clones obtained from a fusion after immunization with haptens of partial sequences of hemoglobin containing histidine-sulfur mustard adduct. Supernatants of cultures in a 1:5 dilution were assayed in a direct ELISA on keratin from human callus treated with 0 μ M or 50 μ M sulfur mustard. Fluorescence (in arbitrary units) is presented as a measure for antibody binding.

| clone | Peptide* used for immunization | Antibody response against keratin exposed to sulfur mustard solution of | |
|----------|--------------------------------|---|------------|
| | | 0 μ M | 50 μ M |
| D5-2E9 | 1 | 228 | 2628 |
| 4A3-1B3 | 3 | 238 | 2891 |
| 4A3-1C2 | 3 | 246 | 2668 |
| 4A3-2A10 | 3 | 299 | 2943 |
| 4A3-2B7 | 3 | 293 | 2996 |
| 4A3-2F4 | 3 | 327 | 2969 |
| 4A3-2F6 | 3 | 240 | 2498 |
| 4A3-1A7 | 3 | 160 | 1623 |
| 4A3-2G7 | 3 | 135 | 1578 |
| 4A3-2E8 | 3 | 397 | 3274 |

*These peptides (1: AFSDGLA(N1/N3-HETE)HLDNLK, corresponding with residues 70-82 of human β -globin; 3: L(N1/N3-HETE)HVDPENFRLLGNVK, corresponding with residues 96-109 of human β -globin) have been described in detail previously (12).

Some clones specific for alkylated keratin (see Table 3) showed also a weak affinity for alkylated human serum albumin. Unfortunately, this result could not be reproduced.

IV.5 Characterization of monoclonal antibodies directed against sulfur mustard adducts to proteins

IV.5.1 Antibodies against sulfur mustard adducts to keratin

Nine clones producing antibodies against sulfur mustard adducts to keratin were selected for further characterization and subcloning. As described previously, 32 monoclonal clones, all originating from clone 1.3C2, have been selected of which antibodies showed specificity against keratins isolated from human callus treated with 50 μ M sulfur mustard (12).

Antibodies of one clone, 1H10, were characterized further. Cross-reactivity toward the 3 hapten peptides (GVVSTH(N- ω -HETE)QQVLRTKNK (1); GIQ(N- ω -HETE)QVTVNQSLTPLNK (2) and GVM(N- ω -HETE)NVHDGKVVSTHEK (3)) and the corresponding nascent peptides was assessed in a competitive ELISA (Table 5).

Table 5. Antibody specificity of clone 1H10 obtained from a mouse immunized with peptide 1+2

| 50% inhibition in competitive ELISA with | | |
|--|------------------------------------|-----------------------------|
| | sulfur mustard-peptide (nmol/well) | Nascent-peptide (nmol/well) |
| Peptide 1 | >20 | >20 |
| Peptide 2 | 1.4 | 6.5 |
| Peptide 3 | 7.6 | >20 |

With 1.4 nmol/well of peptide 2 50% inhibition was observed, whereas with the corresponding nascent peptide 6.5 nmol/well was required to achieve the same extent of inhibition. Peptide 3, which was not used for the immunization resulting in clone 1H10, also showed some cross-reactivity (50% inhibition with 7.6 nmol/well). All other peptides tested, including peptide 1 used for the immunization, did not show any cross-reactivity in the concentration range tested, *i.e.*, 50% inhibition is not achieved at amounts less than 20 nmol/well.

IV.6 Development of the immunoslotblot assay for the detection of sulfur mustard adducts to proteins

IV.6.1 Sulfur mustard adducts to hemoglobin

As described previously, several clones of which the antibodies recognized alkylated hemoglobin were obtained from mice immunized with an alkylated peptide, *i.e.*, N-acetyl-S-HETE-cys₉₃ through leu₁₀₆-lys of the β -chain of hemoglobin (4). One of these, 3H6, was further characterized. These antibodies recognized sulfur mustard-modified hemoglobin in a dose-dependent way. It appeared that exposure of human hemoglobin to 50 μ M sulfur mustard was detectable in a direct ELISA (4). However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have attempted to apply these antibodies to an immunoslotblot assay for alkylated hemoglobin, but so far without lowering the minimum detectable concentration.

IV.6.2 Sulfur mustard adducts to human keratin

We applied the immunoslotblot assay, developed for the detection of N7-HETE-Gua in DNA, on sulfur mustard adducts with keratin using the antibodies 1H10 directed against these adducts. Due to the complications with the contamination with small amounts of SDS in the keratin samples the results as described in the previous annual report could not be reproduced to the same extent. We decided first to make clear how to circumvent the problems with SDS before to repeat those experiments.

V DISCUSSION

Development of a Standard Operating Procedure for determination of sulfur mustard protein adducts; mass spectrometric or fluorescence-based analysis of an adducted tripeptide from albumin (part A.; t.o. 1-9)

On the basis of the findings obtained in the first year of the cooperative agreement, *i.e.*,

- considerably shortening of the procedure for albumin isolation by affinity chromatography
- the convenient use of an internal standard, *i.e.*, plasma isolated from blood that had been exposed to sulfur mustard-d8

a tentative Standard Operating Procedure has been drafted for the analysis of the modified tripeptide (S-HETE)Cys-Pro-Phe, resulting from sulfur mustard-modified albumin.

In the future, we plan to use columns with immobilized pronase. This might enable the construction of an automated system of (reactor) columns, coupled to a tandem mass spectrometer, in which an unprocessed plasma sample can be introduced. This would be highly convenient for use under field laboratory conditions, and more importantly, would speed up the actual diagnosis.

In preliminary *in vitro* experiments with [¹⁴C]-labelled sulfur mustard we had found in the first year of the cooperative grant agreement that the free cysteine-34 residue in rat albumin is also prone to alkylation by sulfur mustard. It was found by LC-tandem MS analysis that a tripeptide (S-HETE)Cys-Pro-Tyr was formed after pronase digestion. The level of alkylation at the particular cysteine residue was approximately 5%. On the basis of these results we believe that the rat is a good animal for studying the persistence of the adduct. Rats were exposed (*i.v.*; 0.3 mg/kg) to sulfur mustard and at certain time points the animals were killed and their blood was collected. After addition of internal standard, albumin was isolated from the plasma by affinity chromatography. Globin was isolated from the erythrocytes by precipitation in acetone/HCl. Subsequently, the albumin samples were treated with pronase and the digests were analyzed by means of LC-tandem MS. Initially, an increase in adduct level could be observed. Subsequently, the adduct level decreased rapidly. The observed half-life time of sulfur mustard alkylated albumin was 2 days, which is in accordance with literature values for albumin adducts (1-3 days; see for instance Troester *et al.* (26)). The albumin adduct was no longer detectable after 7 days after the exposure.

The corresponding globin samples were analyzed for the presence of adducts to the N-terminal valine residues. Remarkably, the adduct level clearly increased during the first two days, which implicates that there is still free sulfur mustard present during that time, which causes accumulating damage. Interestingly, Langenberg *et al.* (27) already observed a long terminal half-life of sulfur mustard in blood after intravenous exposure of hairless guinea pigs to sulfur mustard. The slow elimination of unchanged sulfur mustard was already observed by Maisonneuve *et al.* (28), after *i.v.* administration of the agent to rats. Obviously, the long terminal half-life of sulfur mustard in blood is the result of redistribution from the tissues into the blood. Probably, sulfur mustard accumulates in the adipose tissue after which it can slowly re-enter the blood stream. Also, it might accumulate in the cell membranes, *e.g.*, of the red blood cell, from which it is slowly released. This phenomenon was also observed in a previous Cooperative Agreement (DAMD17-97-2-7002), in which a marmoset was exposed to sulfur mustard. However, this experiment was performed with only one animal. The N-terminal valine adduct level decreased more or less linearly, in accordance with the life-time of the erythrocyte of the rat (reported life-time of rat erythrocyte 65 days (29)). The adduct to the N-terminal valine could still be detected after 28 days. Since the aim of these experiments was to study the persistence of the albumin adducts *in vivo*, we have used *i.v.* administration (as was also stated in the Statement of Work) of sulfur mustard for these initial experiments in order to be sure that well-defined amounts of sulfur mustard were available for adduct formation. Nonetheless, there are various clues that sulfur mustard will also enter the blood stream during more 'realistic' battlefield exposure scenarios, *e.g.*, by inhalation or by

percutaneous exposure. For instance, Langenberg *et al* (27) reported measurable concentrations of DNA-sulfur mustard adducts in blood of hairless guinea pigs after nose-only exposure to sulfur mustard (dose corresponding to 1 LCt50). It is evident that in such case also albumin adducts will be formed since, in contrast to DNA, albumin is not enclosed within a cell membrane. In the same study, DNA adducts could be analyzed after percutaneous exposure (1 Lct50) of hairless guinea pigs to sulfur mustard vapor; even intact sulfur mustard could be analyzed in this case up to concentrations of 12 ng/ml (75 nM) blood. Such concentrations, and also lower concentrations, will definitely give rise to the formation of measurable concentrations of albumin adducts.

It will also be attempted in the third year of the grant period to derivatize (S-HETE)Cys-Pro-Phe with FAM succinimidyl ester for analysis with CE-LIF, analogous to the histidine adducts (see below).

Development of a Standard Operating Procedure for determination of sulfur mustard protein adducts; analysis of N1/N3-HETE-histidine (*part B, t.o. 10-13*).

Although histidine adduct are the most abundant adducts resulting after exposure of proteins to sulfur mustard, there is no sensitive method for their analysis available. For the histidine adducts an isolation procedure from acidic hydrolysates was developed based on cation exchange chromatography. Unfortunately, this method proved to be rather irreproducible until now. In order to improve the isolation procedure for the histidine adducts, it will be attempted in the third year of the agreement to semi-preparatively separate underivatized N1/N3-HETE-histidine by means of reversed phase HPLC.

We also proposed to study the feasibility of fluorescence detection of sulfur mustard-histidine adducts. Although HPLC with fluorescence detection is much less specific than mass spectrometric detection, it has the advantage that yoctomole amounts of amino acid derivatives can be analyzed. In the current grant period a number of fluorescent derivatives of the histidine-adduct were synthesized, which will be used as reference compounds. The carboxyfluoresceine (FAM) derivative of N1/N3-histidine was chosen for further analyses, because it can be prepared quite easily using a commercially available reagent and it proved to be a stable derivative, in contrast to the FITC derivative that slowly rearranged. Capillary electrophoresis with LIF detection gave the best results. Currently, the detection limit of the FAM derivatives of N1/N3-HETE-histidine was determined to be 20-25 pg/ml, which is excellent in comparison with the GC-MS analysis. However, whether this method can be applied to "real" samples, i.e., processed acidic digests of globin or albumin, remains to be seen, and will be investigated in the third year of the grant period. Obviously, one of the disadvantages of not following an MS-based approach is that it is hard to distinguish between all the fluorescent derivatives, especially when the compound of interest is only present in low amounts compared to other compounds as is definitely the case when analyzing adducts. Nonetheless, the CE-LIF approach will be pursued further in the third year of the grant period.

Development of a Standard Operating Procedure for determination of sulfur mustard protein adduct; comparison of the albumin - tripeptide method with the histidine adduct method (*part C, t.o. 14-18*)

Comparison of the two methods, i.e., tripeptide adduct and histidine adduct method reveals the following. The disadvantages of the histidine-adduct method are obvious. In order to release the adduct from the protein, an acidic hydrolysis step (with a duration of 16 h) is required that results in a mixture of amino acids that have basically identical properties. Subsequently, laborious ion-exchange and derivatization steps are required in order to differentiate between the various components. Furthermore, the ion-exchange work-up step

was not readily reproducible. The procedure for analysis of the tripeptide adduct (S-HETE)Cys-Pro-Phe originating from albumin is a very rapid, straightforward method for diagnosis of exposure to sulfur mustard, and is in our view at this moment superior to the method for determination of histidine adducts. The work-up of the plasma sample is minimal, no derivatization is required, and the method can probably be automated. Also, in this case it will be attempted to analyze the tripeptide as its FAM derivative by means of CE-LIF.

Immunochemical protein adduct analysis (Part D, t.o. 19-24)

The main advantage of detection of adducts to proteins over those to DNA is the expected much longer half-life of the protein adducts. Therefore, antibodies were raised against (S-HETE)-cysteine in partial sequences of human hemoglobin in our previous studies (4). However, the minimum detectable concentration obtained for *in vitro* exposure of human blood with these antibodies was only 50 μ M of sulfur mustard. Consequently, further exploratory research on immunochemical assays of protein adducts was a major topic of the current study.

Investigations were performed on three proteins, *i.e.*, hemoglobin, albumin, and keratin. The accessibility of the adducts for immunochemical analysis is supposed to increase in this order, *i.e.*, hemoglobin is enclosed in erythrocytes, albumin is freely circulating in the plasma, whereas keratins in the skin are directly accessible from the environment for sulfur mustard and for reagents.

Immunochemical analysis of hemoglobin adducts;

As described in the previous annual report several antibodies were raised against adducts of sulfur mustard to N-terminal valine, histidin and cystein residues of hemoglobin. Further analysis of these antibodies and application in immunoslotblot assay was postponed to the third year due to the efforts in attempts to solve the problems with the selection of clones producing antibodies against sulfur mustard adducts to keratin (see section IV-3.4.)

Immunochemical analysis of keratin adducts

In addition to the respiratory tract, the skin is a major target for vesicants such as sulfur mustard. Proteins in the skin, particularly those in the stratum corneum, are readily accessible to agents. Since keratin is the most abundant protein in stratum corneum and epidermis, methods for retrospective detection of skin exposure to sulfur mustard were developed in the present study.

Keratins (MW 40-70 kDa) form the backbone of the intermediate filaments (IFs) in epithelial tissues (30, 31). In basal epidermal cells almost 30% of all synthesized proteins are keratins. Their structures are closely related and can be represented by a central α -helix rich domain (length 300-350 residues) flanked on either side by non-helical domains of variable size and chemical character. The helical segments contain heptad repeats of hydrophobic residues. In addition, a conserved periodic distribution of acidic (aspartic acid, glutamic acid) and basic (arginine, histidine and lysine) amino acids is found in IFs. The termini contain *inter alia* inexact repeats of glycine and (phospho)serine residues. Amino acid sequences of a number of human keratins have been documented (30). Most data indicate that the end domains are predominantly located on the surface of the IFs.

For development of an immunochemical assay, two partial end domain sequences of keratin K14 and one partial end domain sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies, as earlier described (12). This approach appeared to be very successful for raising antibodies (15). Fusions from mice immunized with only one peptide or with a mixture of two or three peptides resulted in specific antibodies to sulfur mustard adducts in keratin isolated from human callus. Antibody 1H10 was raised against adducts present in end domains of keratins K5, K14 and K1, of which K5 and K14 are two of the most important keratins in basal cells and K1 is the main keratin in the stratum corneum. The

competitive ELISA turned out that antibody 1H10 only recognised sulfur mustard adducts to peptide 2 which contained amino acid sequences present both in end domains of K1 and K5. Furthermore it appeared also important to apply freshly prepared supernatants of cultures of clones producing specific antibodies.

Unfortunately, some problems arose with antibodies specific for alkylated keratin, with regard to the reproducibility of the assay. These problems will be studied in more detail in the third year of the grant period.

VI KEY RESEARCH ACCOMPLISHMENTS OBTAINED IN THIS GRANT PERIOD

1. The use of an internal standard, *i.e.*, albumin isolated from human blood exposed to sulfur mustard- d_8 , has been worked out completely.
2. The particular albumin sulfur mustard adduct can be detected in the rat *in vivo* at least 7 days after the exposure, with a half-life of approximately 2 days for the albumin adduct, which is in accordance with literature values for rat albumin.
3. The adduct to the N-terminal valine residue in hemoglobin can be detected in the rat *in vivo* at least 28 days after the exposure.
4. The level of N-terminal valine adduct increases during the first 2-3 days after the exposure, indicating the presence of intact sulfur mustard during that period.
5. Several fluorescent derivatives of the histidine adducts have been prepared; the 5/6-carboxyfluorescein (FAM) derivatives could be detected at a level of 20-25 pg/ml with capillary electrophoresis – laser induced fluorescence detection.
6. On the basis of the present results, it can be expected that a Standard Operating Procedure for diagnosis of exposure to low levels of sulfur mustard will be based on LC-tandem MS analysis of the tripeptide (S-HETE)Cys-Pro-Phe originating from albumin; a tentative SOP was drafted for this method.
7. An improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin.

VII REPORTED OUTCOMES (from beginning of cooperative agreement)

Publications

NOORT, D., FIDDER, A., HULST, A.G., AND LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

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NOORT, D., FIDDER, A., HULST, A.G., AND LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

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VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., AND LANGENBERG, J.P. (2002) Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Proceedings of the meeting of NATO TG-004, November 2002, Oslo, Norway.

NOORT, D. (2003) Use of LC tandem MS techniques in identification and sensitive detection of covalent adducts of xenobiotics with proteins, Drug Metabolism Reviews, 35 (S 1), 3.

Presentations

Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Presented by G.P. Van der Schans at meeting of NATO TG-004, November 2002, Oslo, Norway.

Use of LC tandem MS techniques in identification and sensitive detection of covalent adducts of xenobiotics with proteins". Presented by D. Noort at "8th European ISSX Meeting", Dijon, France. April 27 – May 1, 2003.

VIII CONCLUSIONS

1. The albumin tripeptide assay, based on our previous finding that upon pronase treatment of sulfur mustard alkylated albumin a tripeptide cysteine(S-2-hydroxyethylthioethyl)-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) results which has favorable mass spectrometric properties, is a very fast and sensitive assay for biomonitoring of exposure to sulfur mustard.
2. After pronase treatment of rat albumin, isolated from rats exposed to [^{14}C]-labelled sulfur mustard *in vivo*, the tripeptide (S-HETE)Cys-Pro-Tyr could be analyzed in an analogous way as performed for (S-HETE)Cys-Pro-Phe.
3. The albumin adduct was not longer detectable in the rat 7 days after the exposure.
4. The half-life time of sulfur mustard - alkylated albumin was 2 days, which is in accordance with literature values for other albumin adducts formed in rats.
5. The corresponding globin samples were analyzed for the presence of adducts to the N-terminal valine residues. The adduct to the N-terminal valine could still be detected after 28 days.
6. The globin adduct level clearly increased during the first 2-3 days, which implicates that there is still free sulfur mustard present during that time, which causes accumulating damage. Subsequently, the adduct level decreased more or less linearly, in accordance with the life-time of the erythrocyte of the rat.
7. For detection with laser-induced fluorescence, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared. The most convenient derivative turned out to be the 5/6-carboxyfluoresceine derivative, which could be analyzed at a level down to 20-25 pg/ml with capillary electrophoresis - laser induced fluorescence (CE-LIF) detection.
8. In the current grant the screening procedure for the selection of monoclonal antibodies against adducts of sulfur mustard with proteins (globin, albumin and keratin) has been improved. The most suitable antibodies are used for the development of an immunoslotblot assay for retrospective detection of exposure to sulfur mustard.
9. So far the best results were obtained with the detection of sulfur mustard adducts to keratin isolated from human callus exposed to sulfur mustard. Unfortunately, some problems arose with antibodies specific for alkylated keratin, with regard to the reproducibility of the assay. These problems will be studied in more detail in the third year of the grant period.

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XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE
AGREEMENT

Dr. D. Noort
Dr. G.P. van der Schans
Dr. B.L.M. van Baar
Mr. A. Fidder
Mrs. R.H. Mars-Groenendijk
Mr. A.G. Hulst
Mrs. S. de Kant
Mrs. C.E.A.M. Degenhardt-Langelaan
Mr. E. Wils
Mr. S.H. van Krimpen